

**PATENT APPLICATION**

**NEW INTERFERON  $\beta$ -LIKE MOLECULES**

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**CROSS-REFERENCES TO RELATED APPLICATIONS**

Pursuant to 35 USC 119 and 120, and any other applicable statute or rule, this application claims the benefit of and priority from each of the following Application Numbers/filing dates: Denmark Patent Application No. PA 1999 01197, filed August 27, 1999; USSN 60/160,782, filed October 21, 1999; Denmark Patent Application No. PA 1999 01691, filed November 26, 1999; USSN 60/169,077, filed December 6, 1999; Denmark Patent Application No. PA 2000 00194, filed February 7, 2000; Denmark Patent Application No. PA 2000 00363, filed March 7, 2000; USSN 60/189,599, filed March 15, 2000; Denmark Patent Application No. PA 2000 00642, filed April 14, 2000; and USSN 60/202,248, filed May 5, 2000, the disclosures of which are incorporated by reference.

**BACKGROUND OF THE INVENTION**

Interferons are important cytokines characterized by antiviral, antiproliferative, and immunomodulatory activities. These activities form a basis for the clinical benefits that have been observed in a number of diseases, including hepatitis, various cancers and multiple sclerosis. The interferons are divided into the type I and type II classes. Interferon  $\beta$  belongs to the class of type I interferons, which also includes interferons  $\alpha$ ,  $\tau$  and  $\omega$ , whereas interferon  $\gamma$  is the only known member of the distinct type II class.

Human interferon  $\beta$  is a regulatory polypeptide with a molecular weight of 22 kDa consisting of 166 amino acid residues. It can be produced by most cells in the body, in particular fibroblasts, in response to viral infection or exposure to other biologics. It binds to a multimeric cell surface receptor, and productive receptor binding results in a cascade of

5 intracellular events leading to the expression of interferon  $\beta$  inducible genes which in turn produces effects which can be classified as antiviral, antiproliferative and immunomodulatory.

The amino acid sequence of human interferon  $\beta$  was reported by Taniguchi, Gene 10:11-15, 1980, and in EP 83069, EP 41313 and US 4686191.

Crystal structures have been reported for human and murine interferon  $\beta$ ,  
10 respectively (Proc. Natl. Acad. Sci. USA 94:11813-11818, 1997. J. Mol. Biol. 253:187-207, 1995). They have been reviewed in Cell Mol. Life Sci. 54:1203-1206, 1998.

Relatively few protein-engineered variants of interferon  $\beta$  have been reported (WO 9525170, WO 9848018, US 5545723, US 4914033, EP 260350, US 4588585, US 4769233, Stewart et al, DNA Vol 6 no2 1987 pp. 119-128, Runkel et al, 1998, Jour. Biol. Chem. 273, No.  
15 14, pp. 8003-8008).

Expression of interferon  $\beta$  in CHO cells has been reported (US 4966843, US 5376567 and US 5795779).

Redlich et al, Proc. Natl. Acad. Sci., USA, Vol. 88, pp. 4040-4044, 1991 disclose immunoreactivity of antibodies against synthetic peptides corresponding to peptide stretches of  
20 recombinant human interferon  $\beta$  with the mutation C17S.

*Sub  $\beta$*  Interferon  $\beta$  molecules with a particular glycosylation pattern and methods for their preparation have been reported (EP 287075 and EP 539300).

Various references disclose modification of polypeptides by polymer conjugation or glycosylation. Polymer modification of native interferon  $\beta$  or a C17S variant thereof has been  
25 reported (EP 229108, US 5382657, EP 593868, US 4917888 and WO 99/55377). US 4,904,584 discloses PEGylated lysine depleted polypeptides, wherein at least one lysine residue has been deleted or replaced with any other amino acid residue. WO 99/67291 discloses a process for conjugating a protein with PEG, wherein at least one amino acid residue on the protein is deleted and the protein is contacted with PEG under conditions sufficient to achieve conjugation to the  
30 protein. WO 99/03887 discloses PEGylated variants of polypeptides belonging to the growth hormone superfamily, wherein a cysteine residue has been substituted with a non-essential amino acid residue located in a specified region of the polypeptide. Interferon  $\beta$  is mentioned as one example of a polypeptide belonging to the growth hormone superfamily. WO 00/23114 discloses glycosylated and pegylated interferon  $\beta$ . WO 00/23472 discloses interferon  $\beta$  fusion

5 proteins. WO 00/26354 discloses a method of producing a glycosylated polypeptide variant with reduced allergenicity, which as compared to a corresponding parent polypeptide comprises at least one additional glycosylation site. US 5,218,092 discloses modification of granulocyte colony stimulating factor (G-CSF) and other polypeptides so as to introduce at least one additional carbohydrate chain as compared to the native polypeptide. Interferon  $\beta$  is mentioned  
10 as one example among many polypeptides which allegedly can be modified according to the technology described in US 5,218,092.

Commercial preparations of interferon  $\beta$  are sold under the names Betaseron® (also termed interferon  $\beta$ 1b, which is non-glycosylated, produced using recombinant bacterial cells, has a deletion of the N-terminal methionine residue and the C17S mutation), and  
15 Avonex™ and Rebif® (also termed interferon  $\beta$ 1a, which is glycosylated, produced using recombinant mammalian cells) for treatment of patients with multiple sclerosis, and have been shown to be effective in reducing the exacerbation rate. More patients treated with these interferon  $\beta$  agents remain exacerbation-free for prolonged periods of time as compared with placebo-treated patients. Furthermore, the accumulation rate of disability is reduced (Neurol.  
20 51:682-689, 1998).

Comparison of interferon  $\beta$ 1a and  $\beta$ 1b with respect to structure and function has been presented in Pharmaceut. Res. 15:641-649, 1998.

Interferon  $\beta$  is the first therapeutic intervention shown to delay the progression of multiple sclerosis, a relapsing then progressive inflammatory degenerative disease of the central  
25 nervous system.

Its mechanism of action, however, remains largely unclear. It appears that interferon  $\beta$  has inhibitory effects on the proliferation of leukocytes and antigen presentation. Furthermore, interferon  $\beta$  may modulate the profile of cytokine production towards an anti-inflammatory phenotype. Finally, interferon  $\beta$  can reduce T-cell migration by inhibiting the  
30 activity of T-cell matrix metalloproteases. These activities are likely to act in concert to account for the mechanism of interferon  $\beta$  in MS (Neurol. 51:682-689, 1998).

In addition, interferon  $\beta$  may be used for the treatment of osteosarcoma, basal cell carcinoma, cervical dysplasia, glioma, acute myeloid leukemia, multiple myeloma, Hodgkin's disease, breast carcinoma, melanoma, and viral infections such as papilloma virus, viral hepatitis,

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5 herpes genitalis, herpes zoster, herpetic keratitis, herpes simplex, viral encephalitis, cytomegalovirus pneumonia, and rhinovirus.

Various side effects are associated with the use of current preparations of interferon  $\beta$ , including injection site reactions, fever, chills, myalgias, arthralgias, and other flu-like symptoms (Clin. Therapeutics, 19:883-893, 1997).

10 In addition, 6-40% of patients develop neutralizing antibodies to interferon  $\beta$  (Int. Arch. Allergy Immunol. 118:368-371, 1999). It has been shown that development of interferon  $\beta$ -neutralizing antibodies decreases the biological response to interferon  $\beta$ , and cause a trend towards decreased treatment efficacy (Neurol. 50:1266-1272, 1998). Neutralizing antibodies will likely also impede the therapeutic utility of interferon  $\beta$  in connection with treatment of other  
15 diseases (Immunol. Immuther. 39:263-268, 1994).

Given the magnitude of side effects with current interferon  $\beta$  products, their association with frequent injection, the risk of developing neutralizing antibodies impeding the desired therapeutic effect of interferon  $\beta$ , and the potential for obtaining more optimal therapeutic interferon  $\beta$  levels with concomitant enhanced therapeutic effect, there is clearly a  
20 need for improved interferon  $\beta$ -like molecules.

### SUMMARY OF THE INVENTION

This application discloses improved interferon  $\beta$  molecules providing one or more of the aforementioned desired benefits. In particular conjugates are disclosed that exhibit interferon  $\beta$  activity and comprise at least one non-polypeptide moiety covalently attached to an  
25 interferon  $\beta$  polypeptide that comprises an amino acid sequence that differs from that of wildtype human interferon  $\beta$  with the amino acid sequence shown in SEQ ID NO 2 in at least one amino acid residue selected from an introduced or removed amino acid residue comprising an attachment group for the non-polypeptide moiety. Conjugates of the present invention have a number of improved properties as compared to human interferon  $\beta$ , including reduced  
30 immunogenicity, increased functional *in vivo* half-life, increased serum half-life, and/or increased bioavailability. Consequently, the conjugate of the invention offers a number of advantages over the currently available interferon  $\beta$  compounds, including longer duration between injections, fewer side effects, and/or increased efficiency due to reduction in antibodies. Moreover, higher doses of active protein and thus a more effective therapeutic response may be

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5 obtained by use of a conjugate of the invention. Furthermore, conjugates of the invention have demonstrated significantly reduced cross-reactivity with sera from patients treated with currently available interferon  $\beta$  products as defined hereinbelow.

10 In one aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising at least one first non-polypeptide moiety covalently attached to an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in at least one introduced and at least one removed amino acid residue comprising an attachment group for said first non-polypeptide moiety.

15 In another aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising at least one first non-polypeptide moiety conjugated to at least one lysine residue of an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in at least one introduced and/or at least one removed lysine residue.

20 In yet another aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising at least one first non-polypeptide moiety conjugated to at least one cysteine residue of an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from at least one introduced cysteine residue into a position that in wild-type human interferon  $\beta$  is occupied by a surface exposed amino acid residue.

25 In yet another aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising at least one first non-polypeptide moiety having an acid group as an attachment group, which moiety is conjugated to at least one aspartic acid or glutamic acid residue of an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in at least one introduced and/or at least one removed aspartic acid or glutamic acid residue.

30 In yet another aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising at least one polymer molecule and at least one sugar moiety covalently attached to an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in

(a) at least one introduced and/or at least one removed amino acid residue comprising an attachment group for the polymer molecule, and

5 (b) at least one introduced and/or at least one removed amino acid residue comprising an attachment group for the sugar moiety,

provided that when the attachment group for the polymer molecule is a cysteine residue, and the sugar moiety is an N-linked sugar moiety, a cysteine residue is not inserted in such a manner that an N-glycosylation site is destroyed.

10 In yet another aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in at least one introduced glycosylation site, the conjugate further comprising at least one un-PEGylated sugar moiety attached to an introduced glycosylation site.

15 In yet another aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in that a glycosylation site has been introduced or removed by way of introduction or removal of amino acid residue(s) constituting a part of a glycosylation site in a position that in wildtype human interferon  $\beta$  is occupied by a surface exposed amino acid residue.

20 In a still further aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising a sugar moiety covalently attached to an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in at least one removed glycosylation site.

25 In still further aspects the invention relates to means and methods for preparing a conjugate or interferon  $\beta$  polypeptide for use in the invention, including nucleotide sequences and expression vectors encoding the polypeptide as well as methods for preparing the polypeptide or the conjugate.

30 In final aspects the invention relates to a therapeutic composition comprising a conjugate of the invention, to a conjugate or composition of the invention for use in therapy, to the use of a conjugate or composition in therapy or for the manufacture of a medicament for treatment of diseases.

#### **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 illustrates the antiviral activity of a conjugate of the invention,

5 Figure 2 depicts the yield of interferon  $\beta$  production obtained according to  
Example 8.

## DETAILED DISCUSSION

### DEFINITIONS

10 In the context of the present application and invention the following definitions  
apply:

15 The term "conjugate" (or interchangeably "conjugated polypeptide") is intended  
to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the  
covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties. The  
term covalent attachment means that the polypeptide and the non-polypeptide moiety are either  
directly covalently joined to one another, or else are indirectly covalently joined to one another  
through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or  
moieties using an attachment group present in the polypeptide. Preferably, the conjugate is  
soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as  
blood. Examples of conjugated polypeptides of the invention include glycosylated and/or  
20 PEGylated polypeptides. The term "non-conjugated polypeptide" may be used about the  
polypeptide part of the conjugate.

25 The term "non-polypeptide moiety" is intended to indicate a molecule that is  
capable of conjugating to an attachment group of the polypeptide of the invention. Preferred  
examples of such molecule include polymer molecules, sugar moieties, lipophilic compounds, or  
organic derivatizing agents. When used in the context of a conjugate of the invention it will be  
understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugate  
through an attachment group of the polypeptide.

30 The term "polymer molecule" is defined as a molecule formed by covalent  
linkage of two or more monomers, wherein none of the monomers is an amino acid residue,  
except where the polymer is human albumin or another abundant plasma protein. The term  
"polymer" may be used interchangeably with the term "polymer molecule". The term is intended  
to cover carbohydrate molecules attached by *in vitro* glycosylation, i.e. a synthetic glycosylation  
performed *in vitro* normally involving covalently linking a carbohydrate molecule to an  
attachment group of the polypeptide, optionally using a cross-linking agent. Carbohydrate



5 molecules attached by *in vivo* glycosylation, such as N- or O-glycosylation (as further described below)) are referred to herein as "a sugar moiety". Except where the number of non-polypeptide moieties, such as polymer molecule(s) or sugar moieties in the conjugate is expressly indicated every reference to "a non-polypeptide moiety" contained in a conjugate or otherwise used in the present invention shall be a reference to one or more non-polypeptide moieties, such as polymer  
10 molecule(s) or sugar moieties, in the conjugate.

The term "attachment group" is intended to indicate an amino acid residue group of the polypeptide capable of coupling to the relevant non-polypeptide moiety. For instance, for polymer, in particular PEG, conjugation a frequently used attachment group is the  $\epsilon$ -amino group of lysine or the N-terminal amino group. Other polymer attachment groups include a free  
15 carboxylic acid group (e.g. that of the C-terminal amino acid residue or of an aspartic acid or glutamic acid residue), suitably activated carbonyl groups, oxidized carbohydrate moieties and mercapto groups.

For *in vivo* N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting an N-glycosylation site (with the sequence N-X'-S/T/C-X'', wherein X' is any amino acid residue except proline, X'' any amino acid residue that may or may not be identical to X' and preferably is different from proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-  
20 glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present. Accordingly, when the non-polypeptide moiety is an N-linked sugar moiety, the term "amino acid residue comprising an attachment group for the non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the parent polypeptide is to be understood as amino acid residues constituting an N-glycosylation site is/are to be altered in such  
25 a manner that either a functional N-glycosylation site is introduced into the amino acid sequence or removed from said sequence. For an "O-glycosylation site" the attachment group is the OH-group of a serine or threonine residue.

The term "one difference" or "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified  
35 amino acid difference. For instance, in addition to the removal and/or introduction of amino acid

residues comprising an attachment group for the non-polypeptide moiety the interferon  $\beta$  polypeptide may comprise other substitutions that are not related to introduction and/or removal of such amino acid residues. The term "at least one" as used about a non-polypeptide moiety, an amino acid residue, a substitution, etc is intended to mean one or more. The terms "mutation" and "substitution" are used interchangeably herein.

In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) ([www.pdb.org](http://www.pdb.org)) which are based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names e.t.c.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985). CA is sometimes referred to as C $\alpha$ , CB as C $\beta$ . The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues. The terminology used for identifying amino acid positions/substitutions is illustrated as follows: C17 (indicates position #17 occupied by a cysteine residue in the amino acid sequence shown in SEQ ID NO 2). C17S (indicates that the cysteine residue of position 17 has been replaced with a serine). The numbering of amino acid residues made herein is made relative to the amino acid sequence shown in SEQ ID NO 2. "M1del" is used about a deletion of the methionine residue occupying position 1. Multiple substitutions are indicated with a "+", e.g. R71N+D73T/S means an amino acid sequence which comprises a substitution of the arginine residue in position 71 with an asparagine and a substitution of the aspartic acid residue in position 73 with a threonine or serine residue, preferably a threonine residue. T/S as used about a given substitution herein means either a T or a S residue, preferably a T residue.

The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The term "interferon  $\beta$  protein sequence family" is used in its conventional meaning, i.e. to indicate a group of polypeptides with sufficiently homologous amino acid

5 sequences to allow alignment of the sequences, e.g. using the CLUSTALW program. An  
interferon  $\beta$  sequence family is available, e.g. from the PFAM families, version 4.0, or may be  
prepared by use of a suitable computer program such as CLUSTALW version 1.74 using default  
parameters (Thompson et al., 1994, CLUSTAL W: improving the sensitivity of progressive  
multiple sequence alignment through sequence weighting, position-specific gap penalties and  
10 weight matrix choice, Nucleic Acids Research, 22:4673-4680).

The term "polymerase chain reaction" or "PCR" generally refers to a method for  
amplification of a desired nucleotide sequence in vitro, as described, for example, in US  
4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis,  
using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

15 "Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein  
and all such terms should be understood to include progeny resulting from growth or culturing of  
a cell. "Transformation" and "transfection" are used interchangeably to refer to the process of  
introducing DNA into a cell.

20 "Operably linked" refers to the covalent joining of two or more nucleotide  
sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one  
another such that the normal function of the sequences can be performed. For example, the  
nucleotide sequence encoding a presequence or secretory leader is operably linked to a  
nucleotide sequence for a polypeptide if it is expressed as a preprotein that participates in the  
secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it  
25 affects the transcription of the sequence; a ribosome binding site is operably linked to a coding  
sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that  
the nucleotide sequences being linked are contiguous and, in the case of a secretory leader,  
contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction  
sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in  
30 conjunction with standard recombinant DNA methods.

The term "introduce" is primarily intended to mean substitution of an existing  
amino acid residue, but may also mean insertion of an additional amino acid residue. The term  
"remove" is primarily intended to mean substitution of the amino acid residue to be removed by  
another amino acid residue, but may also mean deletion (without substitution) of the amino acid  
35 residue to be removed.

5 The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8<sup>th</sup> Edition, Blackwell) for further definition of immunogenicity). Immunogenicity may be determined by use of any suitable method known in the art, e.g. *in vivo* or *in vitro*, e.g. using the *in vitro* immunogenicity test outlined in the Materials and Methods section below. The term "reduced immunogenicity" is intended to indicate that the conjugate or polypeptide of the present invention gives rise to a measurably lower immune response than a reference molecule, such as wildtype human interferon  $\beta$ , e.g. Rebif or Avonex, or a variant of wild-type human interferon  $\beta$  such as Betaseron, as determined under comparable conditions. When reference is made herein to commercially available interferon  $\beta$  products (i.e. Betaseron, Avonex and Rebif), it should be understood to mean either the formulated product or the interferon  $\beta$  polypeptide part of the product (as appropriate). Normally, reduced antibody reactivity (e.g. reactivity towards antibodies present in serum from patients treated with commercial interferon  $\beta$  products) is an indication of reduced immunogenicity.

20 The term "functional *in vivo* half-life" is used in its normal meaning, i.e. the time at which 50% of a given functionality of the polypeptide or conjugate is retained (such as the time at which 50% of the biological activity of the polypeptide or conjugate is still present in the body/target organ, or the time at which the activity of the polypeptide or conjugate is 50% of the initial value). As an alternative to determining functional *in vivo* half-life, "serum half-life" may be determined, i.e. the time in which 50% of the polypeptide or conjugate molecules circulate in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining functional *in vivo* half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional *in vivo* half-life. Alternative terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The functionality to be retained is normally selected from antiviral, antiproliferative, immunomodulatory or receptor binding activity. Functional *in vivo* half-life and serum half-life may be determined by any suitable method known in the art as further discussed in the Materials and Methods section hereinafter.

35 The polypeptide or conjugate is normally cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, or by specific or unspecific

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5 proteolysis. Clearance taking place by the kidneys may also be referred to as “renal clearance”  
and is e.g. accomplished by glomerular filtration, tubular excretion or tubular elimination.  
Normally, clearance depends on physical characteristics of the conjugate, including molecular  
weight, size (diameter) (relative to the cut-off for glomerular filtration), charge, symmetry,  
shape/rigidity, attached carbohydrate chains, and the presence of cellular receptors for the  
10 protein. A molecular weight of about 67 kDa is considered to be an important cut-off-value for  
renal clearance.

Reduced renal clearance may be established by any suitable assay, e.g. an  
established *in vivo* assay. Typically, the renal clearance is determined by administering a labelled  
(e.g. radiolabelled or fluorescence labelled) polypeptide conjugate to a patient and measuring the  
15 label activity in urine collected from the patient. Reduced renal clearance is determined relative  
to the corresponding non-conjugated polypeptide or the non-conjugated corresponding wild-type  
polypeptide or a commercial interferon  $\beta$  product under comparable conditions.

The term “increased” when used with respect to the functional *in vivo* half-life or  
serum half-life is used to indicate that the relevant half-life of the conjugate or polypeptide is  
20 statistically significantly increased relative to that of a reference molecule, such as an un-  
conjugated wildtype human interferon  $\beta$  (e.g. Avonex™ or Rebif®) or an unconjugated variant  
human interferon  $\beta$  (e.g. Betaseron®) as determined under comparable conditions.

The term “reduced immunogenicity and/or increased functional *in vivo* half-life  
and/or increased serum half-life” is to be understood as covering any one, two or all of these  
25 properties. Preferably, a conjugate or polypeptide of the invention has at least two or these  
properties, i.e. reduced immunogenicity and increased functional *in vivo* half-life, reduced  
immunogenicity and increased serum half-life or increased functional *in vivo* half-life and  
increased serum half-life. Most preferably, the conjugate or polypeptide of the invention has all  
properties.

30 The term “exhibiting interferon  $\beta$  activity” is intended to indicate that the  
polypeptide or conjugate has one or more of the functions of native interferon  $\beta$ , in particular  
human wildtype interferon  $\beta$  with the amino acid sequence shown in SEQ ID NO 2 (which is the  
mature sequence) optionally expressed in a glycosylating host cell or any of the commercially  
available interferon  $\beta$  products. Such functions include capability to bind to an interferon  
35 receptor that is capable of binding interferon  $\beta$  and initiating intracellular signaling from the

receptor, in particular a type I interferon receptor constituted by the receptor subunits IFNAR-2 and IFNAR-1 (Domanski et al., The Journal of Biological Chemistry, Vol. 273, No. 6, pp3144-3147, 1998, Mogensen et al., Journal of Interferon and Cytokine Research, 19: 1069-1098, 1999), and antiviral, antiproliferative or immunomodulatory activity (which can be determined using assays known in the art (e.g. those cited in the following disclosure)). Interferon  $\beta$  activity may be assayed by methods known in the art as exemplified in the Materials and Methods section hereinafter.

The polypeptide or conjugate "exhibiting" or "having" interferon  $\beta$  activity is considered to have such activity, when it displays a measurable function, e.g. a measurable receptor binding and stimulating activity (e.g. as determined by the primary or secondary assay described in the Materials and Methods section). The polypeptide exhibiting interferon  $\beta$  activity may also be termed "interferon  $\beta$  molecule" or "interferon  $\beta$  polypeptide" herein. The term "interferon  $\beta$  polypeptide" is primarily used herein about modified polypeptides of the invention (having introduced or removed attachment groups for the relevant non-polypeptide moiety).

The term "parent interferon  $\beta$ " is intended to indicate the starting molecule to be improved in accordance with the present invention. While the parent interferon  $\beta$  may be of any origin, such as vertebrate or mammalian origin (e.g. any of the origins defined in WO 00/23472), the parent interferon  $\beta$  is preferably wild-type human interferon  $\beta$  with SEQ ID NO 2 or a variant thereof. A "variant" is a polypeptide, which differs in one or more amino acid residues from a parent polypeptide, normally in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues. Examples of wild-type human interferon  $\beta$  include the polypeptide part of Avonex™ or Rebif®. An example of a parent interferon  $\beta$  variant is Betaseron®. Alternatively, the parent interferon  $\beta$  polypeptide may comprise an amino acid sequence, which is a hybrid molecule between interferon  $\beta$  and another homologous polypeptide, such as interferon  $\alpha$ , optionally containing one or more additional substitutions introduced into the hybrid molecule. Such a hybrid molecule may contain an amino acid sequence, which differs in more than 10 amino acid residues from the amino acid sequence shown in SEQ ID NO 2. In order to be useful in the present invention the hybrid molecule exhibits interferon  $\beta$  activity (e.g. as determined in the secondary assay described in the Materials and Methods section herein).

5 The term "functional site" as used about a polypeptide or conjugate of the invention is intended to indicate one or more amino acid residues which is/are essential for or otherwise involved in the function or performance of interferon  $\beta$ , and thus "located at" the functional site. The functional site is e.g. a receptor binding site and may be determined by methods known in the art, preferably by analysis of a structure of the polypeptide complexed to a relevant receptor, such as the type I interferon receptor constituted by IFNAR-1 and IFNAR-2.

### CONJUGATE OF THE INVENTION

As stated above, in a first aspect, the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising at least one first non-polypeptide moiety covalently attached to an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wildtype human interferon  $\beta$  in at least one introduced and at least one removed amino acid residue comprising an attachment group for said first non-polypeptide moiety.

By removing and/or introducing amino acid residues comprising an attachment group for the non-polypeptide moiety it is possible to specifically adapt the polypeptide so as to make the molecule more susceptible to conjugation to the non-polypeptide moiety of choice, to optimize the conjugation pattern (e.g. to ensure an optimal distribution of non-polypeptide moieties on the surface of the interferon  $\beta$  molecule and thereby, e.g., effectively shield epitopes and other surface parts of the polypeptide without significantly impairing the function thereof). For instance, by introduction of attachment groups, the interferon  $\beta$  polypeptide is boosted or otherwise altered in the content of the specific amino acid residues to which the relevant non-polypeptide moiety binds, whereby a more efficient, specific and/or extensive conjugation is achieved. By removal of one or more attachment groups it is possible to avoid conjugation to the non-polypeptide moiety in parts of the polypeptide in which such conjugation is disadvantageous, e.g. to an amino acid residue located at or near a functional site of the polypeptide (since conjugation at such a site may result in inactivation or reduced interferon  $\beta$  activity of the resulting conjugate due to impaired receptor recognition). Further, it may be advantageous to remove an attachment group located closely to another attachment group in order to avoid heterogeneous conjugation to such groups.

It will be understood that the amino acid residue comprising an attachment group for a non-polypeptide moiety, either it be removed or introduced, is selected on the basis of the

5 nature of the non-polypeptide moiety and, in most instances, on the basis of the conjugation  
method to be used. For instance, when the non-polypeptide moiety is a polymer molecule, such  
as a polyethylene glycol or polyalkylene oxide derived molecule, amino acid residues capable of  
functioning as an attachment group may be selected from the group consisting of lysine,  
cysteine, aspartic acid, glutamic acid and arginine. When the non-polypeptide moiety is a sugar  
10 moiety the attachment group is an *in vivo* glycosylation site, preferably an N-glycosylation site.

Whenever an attachment group for a non-polypeptide moiety is to be introduced  
into or removed from the interferon  $\beta$  polypeptide in accordance with the present invention, the  
position of the interferon  $\beta$  polypeptide to be modified is conveniently selected as follows:

The position is preferably located at the surface of the interferon  $\beta$  polypeptide  
15 and more preferably occupied by an amino acid residue which has more than 25% of its side  
chain exposed to the solvent, preferably more than 50% of its side chain exposed to the solvent.  
Such positions have been identified on the basis of an analysis of a 3D structure of the human  
interferon  $\beta$  molecule as described in the Methods section herein.

Alternatively or additionally, the position to be modified is identified on the basis  
20 of an analysis of an interferon  $\beta$  protein sequence family. More specifically, the position to be  
modified can be one, which in one or more members of the family other than the parent  
interferon  $\beta$ , is occupied by an amino acid residue comprising the relevant attachment group  
(when such amino acid residue is to be introduced) or which in the parent interferon  $\beta$ , but not in  
one or more other members of the family, is occupied by an amino acid residue comprising the  
25 relevant attachment group (when such amino acid residue is to be removed).

In order to determine an optimal distribution of attachment groups, the distance  
between amino acid residues located at the surface of the interferon  $\beta$  molecule is calculated on  
the basis of a 3D structure of the interferon  $\beta$  polypeptide. More specifically, the distance  
between the CB's of the amino acid residues comprising such attachment groups, or the distance  
30 between the functional group (NZ for lysine, CG for aspartic acid, CD for glutamic acid, SG for  
cysteine) of one and the CB of another amino acid residue comprising an attachment group are  
determined. In case of glycine, CA is used instead of CB. In the interferon  $\beta$  polypeptide part of  
a conjugate of the invention, any of said distances is preferably more than 8 Å, in particular more  
than 10Å in order to avoid or reduce heterogeneous conjugation.



5 Furthermore, in the interferon  $\beta$  polypeptide part of a conjugate of the invention attachment groups located at the receptor-binding site of interferon  $\beta$  has preferably been removed, preferably by substitution of the amino acid residue comprising such group.

A still further generally applicable approach for modifying an interferon  $\beta$  polypeptide is to shield, and thereby destroy or otherwise inactivate an epitope present in the parent interferon  $\beta$ , by conjugation to a non-polypeptide moiety. Epitopes of human interferon  $\beta$  may be identified by use of methods known in the art, also known as epitope mapping, see, e.g. Romagnoli et al., J. Biol Chem, 1999, 380(5):553-9, DeLisser HM, Methods Mol Biol, 1999, 96:11-20, Van de Water et al., Clin Immunol Immunopathol, 1997, 85(3):229-35, Saint-Remy JM, Toxicology, 1997, 119(1):77-81, and Lane DP and Stephen CW, Curr Opin Immunol, 1993, 5(2):268-71. One method is to establish a phage display library expressing random oligopeptides of e.g. 9 amino acid residues. IgG1 antibodies from specific antisera towards human interferon  $\beta$  are purified by immunoprecipitation and the reactive phages are identified by immunoblotting. By sequencing the DNA of the purified reactive phages, the sequence of the oligopeptide can be determined followed by localization of the sequence on the 3D-structure of the interferon  $\beta$ . Alternatively, epitopes can be identified according to the method described in US 5,041,376. The thereby identified region on the structure constitutes an epitope that then can be selected as a target region for introduction of an attachment group for the non-polypeptide moiety. Preferably, at least one epitope, such as two, three or four epitopes of human recombinant interferon  $\beta$  (optionally comprising the C17S mutation) are shielded by a non-polypeptide moiety according to the present invention. Accordingly, in one embodiment, the conjugate of the invention has at least one shielded epitope as compared to wild type human interferon  $\beta$ , optionally comprising the C17S mutation, including any commercially available interferon  $\beta$ . Preferably, the conjugate of the invention comprises a polypeptide that is modified so as to shield the epitope located in the vicinity of amino acid residue Q49 and/or F111. This may be done by introduction of an attachment group for a non-polypeptide moiety into a position located in the vicinity of (i.e. within 4 amino acid residues in the primary sequence or within about 10Å in the tertiary sequence) of Q49 and/or F111. The 10Å distance is measured between CB's (CA's in case of glycine). Such specific introductions are described in the following sections.

In case of removal of an attachment group, the relevant amino acid residue comprising such group and occupying a position as defined above is preferably substituted with a

5 different amino acid residue that does not comprise an attachment group for the non-polypeptide moiety in question.

In case of introduction of an attachment group, an amino acid residue comprising such group is introduced into the position, preferably by substitution of the amino acid residue occupying such position.

10 The exact number of attachment groups available for conjugation and present in the interferon  $\beta$  polypeptide is dependent on the effect desired to be achieved by conjugation. The effect to be obtained is, e.g., dependent on the nature and degree of conjugation (e.g. the identity of the non-polypeptide moiety, the number of non-polypeptide moieties desirable or possible to conjugate to the polypeptide, where they should be conjugated or where conjugation should be avoided, etc.). For instance, if reduced immunogenicity is desired, the number (and location of) attachment groups should be sufficient to shield most or all epitopes. This is normally obtained when a greater proportion of the interferon  $\beta$  polypeptide is shielded. Effective shielding of epitopes is normally achieved when the total number of attachment groups available for conjugation is in the range of 1-10 attachment groups, in particular in the range of 2-8, such as 3-7.

25 Functional *in vivo* half-life is e.g., dependent on the molecular weight of the conjugate and the number of attachment groups needed for providing increased half-life thus depends on the molecular weight of the non-polypeptide moiety in question. In one embodiment, the conjugate of the invention has a molecular weight of at least 67 kDa, in particular at least 70 kDa as measured by SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), p680-85. Interferon  $\beta$  has a molecular weight of about 20 kDa, and therefore additional about 50kDa is required to obtain the desired effect. This may be, e.g., be provided by 5 10kDa PEG molecules or as otherwise described herein.

30 In order to avoid too much disruption of the structure and function of the parent human interferon  $\beta$  molecule the total number of amino acid residues to be altered in accordance with the present invention (as compared to the amino acid sequence shown in SEQ ID NO 2) typically does not exceed 15. Preferably, the interferon  $\beta$  polypeptide comprises an amino acid sequence, which differs in 1-15 amino acid residues from the amino acid sequence shown in SEQ ID NO 2, such as in 1-8 or in 2-8 amino acid residues, e.g., in 1-5 or in 2-5 amino acid residues from the amino acid sequence shown in SEQ ID NO 2. Thus, normally the interferon  $\beta$

5 polypeptide comprises an amino acid sequence that differs from the amino acid sequence shown  
in SEQ ID NO 2 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues.  
Preferably, the above numbers represent either the total number of introduced or the total number  
of removed amino acid residues comprising an attachment group for the relevant non-  
polypeptide moiety, or the total number of introduced and removed amino acid residues  
10 comprising such group.

In the conjugate of the invention it is preferred that at least about 50% of all  
conjugatable attachment groups, such as at least about 80% and preferably all of such groups are  
occupied by the relevant non-polypeptide moiety. Accordingly, in a preferred embodiment the  
conjugate of the invention comprises, e.g., 1-10 non-polypeptide moieties, such as 2-8 or 3-6.

15 The conjugate of the invention has one or more of the following improved  
properties:

Reduced immunogenicity as compared to wild-type human interferon  $\beta$  (e.g.  
Avonex™ or Rebif®) or to Betaseron®, e.g. a reduction of at least 25%, such as at least 50%,  
and more preferably at least 75%;

20 Increased functional *in vivo* half-life and/or increased serum half-life as compared  
to wild-type human interferon  $\beta$  (e.g. Avonex™ or Rebif®) or to Betaseron®;

Reduced or no reaction with neutralizing antibodies from patients treated with  
wildtype human interferon  $\beta$  (e.g. Rebif® or Avonex™) or with Betaseron®, e.g. a reduction of  
neutralisation of at least 25%, such as of at least 50%, and preferably of at least 75%.

25 The magnitude of the antiviral activity of a conjugate of the invention may not be  
critical, and thus be reduced (e.g. by up to 75%) or increased (e.g. by at least 5%) or equal to that  
of wild-type human interferon  $\beta$  ((e.g. Avonex™ or Rebif®) or to Betaseron®;

Furthermore, the degree of antiviral activity as compared to antiproliferative  
activity of a conjugate of the invention may vary, and thus be higher, lower or equal to that of  
30 wildtype human interferon  $\beta$ .

Conjugate of the invention, wherein the non-polypeptide moiety is a molecule that  
has lysine as an attachment group

In a preferred embodiment the first non-polypeptide moiety has lysine as an  
attachment group, and thus the interferon  $\beta$  polypeptide is one that comprises an amino acid  
35 sequence that differs from that of wildtype human interferon  $\beta$  in at least one introduced and/or

5 at least one removed lysine residue. While the non-polypeptide moiety may be any of those binding to a lysine residue, e.g. the  $\epsilon$ -amino group thereof, such as a polymer molecule, a lipophilic group, an organic derivatizing agent or a carbohydrate moiety, it is preferably any of the polymer molecule mentioned in the section entitled "Conjugation to a polymer molecule", in particular a branched or linear PEG or polyalkylene oxide. Most preferably, the polymer molecule is PEG and the activated molecule to be used for conjugation is SS-PEG, NPC-PEG, 10 aldehyd-PEG, mPEG-SPA, mPEG-SCM, mPEG-BTC from Shearwater Polymers, Inc, SC-PEG from Enzon, Inc., tresylated mPEG as described in US 5,880,255, or oxycarbonyl-oxy-N-dicarboxyimide-PEG (US 5,122,614). Normally, for conjugation to a lysine residue the non-polypeptide moiety has a molecular weight of about 5 or 10 kDa.

15 In one embodiment the amino acid sequence of the interferon  $\beta$  polypeptide differs from that of human wildtype interferon  $\beta$  in at least one removed lysine residue, such as 1-5 removed lysine residues, in particular 1-4 or 1-3 removed lysine residues. The lysine residue(s) to be removed, preferably by replacement, is selected from the group consisting of K19, K33, K45, K52, K99, K105, K108, K115, K123, K134, and K136. The lysine residue(s) 20 may be replaced with any other amino acid residue, but is preferably replaced by an arginine or a glutamine residue in order to give rise to the least structural difference. In particular, the polypeptide part may be one, wherein K19, K45, K52 and/or K123, preferably K19, K45 and/or K123 has/have been replaced with another any other amino acid residue, preferably arginine or glutamine. For instance, the interferon  $\beta$  polypeptide part of a conjugate of the invention 25 comprises a combination of amino acid substitutions selected from the following list:

- K19R+K45R+K123R;
- K19Q+K45R+K123R;
- K19R+K45Q+K123R;
- K19R+K45R+K123Q;
- 30 K19Q+K45Q+K123R;
- K19R+K45Q+K123Q;
- K19Q+K45R+K123Q;
- K19Q+K45Q+K123Q;
- K45R+K123R;
- 35 K45Q+K123R;
- K45Q+K123Q;
- K45R+K123Q;
- K19R+K123R;
- K19Q+K123R;
- 40 K19R+K123Q;

5 K19Q+K123Q;  
K19R+K45R;  
K19Q+K45R;  
K19R+K45Q; or  
K19Q+K45Q.

10 In addition or alternatively to the amino acid substitutions mentioned in the above list the polypeptide part may comprise at least one substitution selected from the group consisting of K33R, K33Q, K52R, K52Q, K99R, K99Q, K105R, K105Q, K108R, K108Q, K115R, K115Q, K134R, K134Q, K136R, and K136Q, e.g. at least one of the following substitutions:

15 K52R+K134R;  
K99R+K136R;  
K33R+K105R+K136R;  
K52R+K108R+K134R;  
K99R+K115R+K136R;  
20 K19R+K33R+K45R+K123R;  
K19R+K45R+K52R+K123R;  
K19R+K33R+K45R+K52R+K123R; or  
K19R+K45R+K52R+K99R+K123R.

In a further embodiment the the amino acid sequence of the interferon  $\beta$  polypeptide differs from that shown in SEQ ID NO 2 in that a lysine residue has been introduced by substitution of at least one amino acid residue occupying a position that in the parent interferon  $\beta$  molecule is occupied by a surface exposed amino acid residue, preferably an amino acid residue having at least 25%, such as at least 50% of its side chain exposed to the surface. Preferably, the amino acid residue to be substituted is selected from the group consisting of N4, F8, L9, R11, S12, F15, Q16, Q18, L20, W22, Q23, G26, R27, L28, E29, Y30, L32, R35, M36, N37, D39, P41, E42, E43, L47, Q48, Q49, T58, Q64, N65, F67, A68, R71, Q72, D73, S75, S76, G78, N80, E81, I83, E85, N86, A89, N90, Y92, H93, H97, T100, L102, E103, L106, E107, E109, D110, F111, R113, G114, L116, M117, L120, H121, R124, G127, R128, L130, H131, E137, Y138, H140, I145, R147, V148, E149, R152, Y155, F156, N158, R159, G162, Y163, R165 and N166 of SEQ ID NO 2.

More preferably, the amino acid sequence of the interferon  $\beta$  polypeptide differs from the amino acid sequence shown in SEQ ID NO 2 in that a lysine residue has been introduced, by substitution, of at least one amino acid residue occupying a position selected from the group consisting of N4, F8, L9, R11, S12, G26, R27, E29, R35, N37, D39, E42, L47, Q48, Q49, A68, R71, Q72, D73, S75, G78, N80, E85, N86, A89, Y92, H93, D110, F111, R113, L116,

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5 H121, R124, G127, R128, R147, V148, Y155, N158, R159, G162 and R165, even more preferably selected from the group consisting of N4, R11, G26, R27, Q48, Q49, R71, D73, S75, N80, E85, A89, Y92, H93, F111, R113, L116, R124, G127, R128, Y155, N158 and G162, and most preferably selected from the group consisting of R11, Q49, R71, S75, N80, E85, A89, H93, F111, R113, L116 and Y155, and most preferably Q49 and F111.

10 In accordance with this embodiment, the interferon  $\beta$  polypeptide comprises a substitution to lysine in one or more of the above positions, in particular in 1-15, such as 1-8 or 1-5, and preferably in at least two positions, such as 2-8 or 2-5 positions.

In a further embodiment the amino acid sequence of the interferon  $\beta$  polypeptide part of a conjugate differs in at least one removed and at least one introduced lysine residue, such as 1-5 or 2-5 removed lysine residues and 1-5 or 2-5 introduced lysine residues. It will be understood that the lysine residues to be removed and introduced preferably are selected from those described in the present section.

In accordance with this embodiment of the invention, the total number of conjugatable lysine residues is preferably in the range of 1-10, such as 2-8 or 3-7.

20 For instance, the interferon  $\beta$  polypeptide part of the conjugate according to this embodiment may comprise at least one of the following substitutions: R11K, Q48K, Q49K, R71K, S75K, N80K, E85K, A89K, H93K, F111K, R113K, L116K and Y155K; more preferably R11K, Q49K, R71K, S75K, N80K, E85K, A89K, H93K, F111K, R113K, L116K and Y155K, in combination with at least one of the substitutions: K19R/Q K33R/Q K45R/Q, K52R/Q, K99R/Q, K105R/Q, K108R/Q, K115R/Q, K123R/Q, K134R/Q, and K136R/Q, wherein R/Q indicates substitution to an R or a Q residue, preferably an R residue. More preferably, the interferon  $\beta$  polypeptide comprises at least one of the following substitutions R11K, Q49K, R71K, S75K, N80K, E85K, A89K, H93K, F111K, R113K, L116K and Y155K, in particular Q49K, F111K and/or N80K, in combination with substitution of at least one of K19, K45, K52 and/or K123, preferably to an R or a Q residue. In particular, the interferon  $\beta$  polypeptide comprises at least one of the substitutions Q49K, F111K and N80K in combination with at least one of the substitutions mentioned above for removal of a lysine residue. For instance, the interferon  $\beta$  polypeptide may comprise the following substitutions:

- 30 Y+Z+K19R+K45R+K123R;  
35 Y+Z+K19Q+K45R+K123R;

- 5 Y+Z+K19R+K45Q+K123R;  
 Y+Z+K19R+K45R+K123Q;  
 Y+Z+K19Q+K45Q+K123R;  
 Y+Z+K19R+K45Q+K123Q;  
 Y+Z+K19Q+K45R+K123Q;  
 10 Y+Z+K19Q+K45Q+K123Q;  
 Y+Z+K45R+K123R;  
 Y+Z+K45Q+K123R;  
 Y+Z+K45Q+K123Q;  
 Y+Z+K45R+K123Q;  
 15 Y+Z+K19R+K123R;  
 Y+Z+K19Q+K123R;  
 Y+Z+K19R+K123Q;  
 Y+Z+K19Q+K123Q;  
 Y+Z+K19R+K45R;  
 20 Y+Z+K19Q+K45R;  
 Y+Z+K19R+K45Q; or  
 Y+Z+K19Q+K45Q, wherein Y is selected from the group of Q49K, F111K, N80K,  
 Q49K+F111K, Q49K+N80K, F111K+N80K and Q49K+F111K+N80K and Z is absent or  
 comprises at least one substitution selected from the group consisting of K33R, K33Q, K52R,  
 25 K52Q, K99R, K99Q, K105R, K105Q, K108R, K108Q, K115R, K115Q, K134R, K134Q,  
 K136R, and K136Q. Preferably, the interferon  $\beta$  polypeptide comprises the following  
 substitution Y+Z+K19R+K45Q+K123R, wherein Y and Z have the above meaning.

More specifically, according to this embodiment the interferon  $\beta$  polypeptide may  
 comprise one of the following substitutions:

- 30 K19R+K45R+F111K+K123R;  
 K19R+K45R+Q49K+F111K+K123R;  
 K19R+K45R+Q49K+K123R;  
 K19R+K45R+ F111K;  
 K19R+K45R+Q49K+F111K;  
 35 K19R+Q49K+K123R;  
 K19R+Q49K+F111K+K123R;  
 K45Q+F111K+K123Q;  
 K45R+Q49K+K123R; or  
 K45R+Q49K+F111K+K123R.

- 40 Especially for expression in a non-glycosylating host such as *E. coli* the interferon  
 $\beta$  polypeptide may contain the substitution N80K or C17S+N80K, optionally in combination  
 with one or more of K19R/Q; K45R/Q; K52R/Q or K123R/Q. The substitution N80K is of  
 particular interest, when the interferon  $\beta$  polypeptide is expressed in a non-glycosylating host

cell, since N80 constitutes part of an inherent glycosylation site of human interferon  $\beta$  and conjugation at such site may mimick natural glycosylation.

Furthermore, it is preferred that the conjugate according to this aspect comprises at least two first non-polypeptide moieties, such as 2-8 moieties. .

Conjugate of the invention wherein the non-polypeptide moiety binds to a cysteine residue

In a still further aspect, the invention relates a conjugate exhibiting interferon  $\beta$  activity and comprising at least one first non-polypeptide conjugated to at least one cysteine residue of an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wildtype human interferon  $\beta$  in that at least one cysteine residue has been introduced, preferably by substitution, into a position that in the parent interferon  $\beta$  molecule is occupied by an amino acid residue that is exposed to the surface of the molecule, preferably one that has at least 25%, such as at least 50% of its side chain exposed to the surface. For instance, the amino acid residue is selected from the group consisting of F8, L9, R11, S12, F15, Q16, Q18, L20, W22, L28, L32, M36, P41, T58, Q64, N65, F67, I83, E85, N86, A89, N90, Y92, H93, H97, T100, L102, E103, L106, M117, L120, H121, R124, G127, R128, L130, H131, H140, I145, R147, V148, E149, R152, Y155, and F156 of SEQ ID NO 2.

Additionally or alternatively, the substitution is preferably performed at a position occupied by a threonine or serine residue. For instance, such position is selected from the group consisting of S2, S12, S13, T58, S74, S75, S76, T77, T82, T100, T112, S118, S119, S139, T144, and T161, more preferably S2, S12, S13, S74, S75, S76, T77, T82, T100, T112, S118, S119, S139, and T144 (side chain surface exposed), still more preferably S2, S12, S75, S76, T82, T100, S119 and S139 (at least 25% of its side chain exposed), and even more preferably S12, S75, T82 and T100 (at least 50% of its side chain exposed). Of the above threonine or serine substitutions, serine substitutions are preferred. Accordingly, in even more preferred embodiments, the position is selected from the group consisting of S2, S12, S13, S74, S75, S76, S118, S119 and S139, more preferably S2, S12, S13, S74, S75, S76, S118, S119 and S139, even more preferably S2, S12, S75, S76, S119 and S139, and still more preferably S12 and S75.

In one embodiment, only one cysteine residue is introduced into the interferon  $\beta$  polypeptide in order to avoid formation of disulphide bridges between two or more introduced cysteine residues. In this connection C17 present in wildtype human interferon  $\beta$  may be



5 removed, preferably by substitution, in particular by substitution with S or A. In another  
embodiment, two or more cysteine residues are introduced, such as 2-6 or 2-4 cysteine residues.  
Preferably, the interferon  $\beta$  polypeptide part of the conjugate according to this embodiment of  
the invention comprises the mutation L47C, Q48C, Q49C, D110C, F111C or R113C, in  
particular only one of these mutations, optionally in combination with the mutation C17S. Also,  
10 the interferon  $\beta$  polypeptide may comprise the substitution C17S+N80C.

While the first non-polypeptide moiety according to this aspect of the invention  
may be any molecule which, when using the given conjugation method has cysteine as an  
attachment group (such as a carbohydrate moiety, a lipophilic group or an organic derivatizing  
agent), it is preferred that the non-polypeptide moiety is a polymer molecule. The polymer  
15 molecule may be any of the molecules mentioned in the section entitled "Conjugation to a  
polymer molecule", but is preferably selected from the group consisting of linear or branched  
polyethylene glycol or polyalkylene oxide. Most preferably, the polymer molecule is VS-PEG.  
The conjugation between the polypeptide and the polymer may be achieved in any suitable  
manner, e.g. as described in the section entitled "Conjugation to a polymer molecule", e.g. in  
20 using a one step method or in the stepwise manner referred to in said section. When the  
interferon  $\beta$  polypeptide comprises only one conjugatable cysteine residue, this is preferably  
conjugated to a first non-polypeptide moiety with a molecular weight of at least 20kDa, either  
directly conjugated or indirectly through a low molecular weight polymer (as disclosed in WO  
99/55377). When the conjugate comprises two or more first non-polypeptide moieties, normally  
25 each of these has a molecular weight of 5 or 10kDa.

Conjugate of the invention wherein the non-polypeptide moiety binds to an acid  
group

In a still further aspect the invention relates to a conjugate exhibiting interferon  $\beta$   
activity and comprising at least one first non-polypeptide moiety having an acid group as the  
30 attachment group, which moiety is conjugated to at least one aspartic acid residue or one  
glutamic acid residue of an interferon  $\beta$  polypeptide, the amino acid sequence of which differs  
from that of wildtype human interferon  $\beta$  in at least one introduced and/or at least one removed  
aspartic acid or glutamic acid residue, respectively. The relevant amino acid residue may be  
introduced in any position occupied by a surface exposed amino acid residue, preferably by an  
35 amino acid residue having more than 25% of its side chain surface exposed. Preferably, at least

5 one amino acid residue occupying a position selected from the group consisting of N4, L5, L6, F8, L9, Q10, R11, S12, S13, F15, Q16, Q18, K19, L20, W22, Q23, L24, N25, G26, R27, Y30, M36, Q46, Q48, Q49, I66, F67, A68, I69, F70, R71, S75, T82, I83, L87, A89, N90, V91, Y92, H93, Q94, I95, N96, H97, K108, F111, L116, L120, K123, R124, Y126, G127, R128, L130, H131, Y132, K134, A135, H140, T144, R147, Y155, F156, N158, R159, G162, Y163 and R165  
10 has been substituted with an aspartic acid residue or a glutamic acid residue.

More preferably, the position is selected from the group consisting of N4, L5, F8, L9, R11, S12, F15, Q16, Q18, K19, W22, Q23, G26, R27, Y30, M36, Q46, Q48, Q49, A68, R71, S75, T82, A89, N90, Y92, H93, N96, H97, K108, F111, L116, L120, K123, R124, G127, R128, L130, H131, K134, A135, H140, Y155, N158, R159, G162, Y163 and R165, such as from  
15 the group consisting of N4, L5, F8, S12, F15, Q16, K19, W22, Q23, R27, Y30, M36, Q46, Q48, Q49, R71, S75, T82, A89, Y92, H93, K108, F111, L116, K123, R124, G127, H131, K134, A135, Y155 and R165, still more preferably from the group consisting of N4, L5, F8, S12, F15, Q16, K19, W22, Q23, R27, Y30, Q46, Q48, Q49, S75, T82, A89, Y92, H93, K108, F111, L116, R124, G127, H131, K134, Y155 and R165, such as from the group consisting of L5, F8, S12, F15, Q16, K19, W22, Q23, Q48, Q49, Y92, H93, R124, G127, H131 and Y155, even more  
20 preferably from the group consisting of S12, Q16, K19, Q23, Q48, Q49, Y92, H93, R124, G127, H131 and Y155, such as from the group consisting of S12, Q16, K19, Q23, Q48, Y92, H93, R124, G127, H131 and Y155, in particular from the group consisting of S12, Q16, K19, Q23, Q48, H93 and H131, even more preferably from the group consisting of S12, Q16, K19, Q48, H93 and H131, and most preferably from the group consisting of Q16 and Q48.  
25

Furthermore, in order to obtain a sufficient number of non-polypeptide moieties it is preferred that that least two aspartic acid residues or at least two glutamic acid residues be introduced, preferably in two positions selected from any of the above lists. Also, it is preferred that the conjugate according to this aspect comprises at least two first non-polypeptide moieties.

30 In case of removal of an amino acid residue, the amino acid sequence of the interferon  $\beta$  polypeptide differs from that of human wildtype interferon  $\beta$  in at least one removed aspartic acid or glutamic acid residue, such as 1-5 removed residues, in particular 1-4 or 1-3 removed aspartic acid or glutamic acid residues. The residue(s) to be removed, preferably by replacement, is selected from the group consisting of D34, D39, D54, D73, D110, E29, E42, E43, E53, E61, E81, E85, E103, E104, E107, E109, E137 and E149. The aspartic acid or  
35

5 glutamic acid residue(s) may be replaced with any other amino acid residue, but is preferably replaced by an arginine or a glutamine residue. first non-polypeptide moiety can be any non-polypeptide moiety with such property, it is presently preferred that the non-polypeptide moiety is a polymer molecule or an organic derivatizing agent having an acid group as an attachment group, in particular a polymer molecule such as PEG, and the conjugate is prepared, e.g., as  
10 described by Sakane and Pardridge, Pharmaceutical Research, Vol. 14, No. 8, 1997, pp 1085-1091. Normally, for conjugation to an acid group the non-polypeptide moiety has a molecular weight of about 5 or 10 kDa.

Conjugate of the invention comprising a second non-polypeptide moiety

In addition to a first non-polypeptide moiety (as described in the preceding  
15 sections), the conjugate of the invention may comprise a second non-polypeptide moiety of a different type as compared to the first non-polypeptide moiety. Preferably, in any of the above described conjugates wherein the first non-polypeptide moiety is, e.g., a polymer molecule such as PEG, a second non- polypeptide moiety is a sugar moiety, in particular an N-linked sugar moiety. While the second non-polypeptide moiety may be attached to a natural glycosylation site  
20 of human interferon  $\beta$ , e.g. the N-linked glycosylation site defined by N80, it is normally advantageous to introduce at least one additional glycosylation site in the interferon  $\beta$  polypeptide. Such site is e.g. any of those described in the immediately preceding section entitled "Conjugate of the invention wherein the non-polypeptide moiety is a sugar moiety". Furthermore, in case at least one additional glycosylation site is introduced this may be  
25 accompanied by removal of an existing glycosylation site as described below.

It will be understood that in order to obtain an optimal distribution of attached first and second non-polypeptide moieties, the interferon  $\beta$  polypeptide may be modified in the number and distribution of attachment groups for the first as well as the second non-polypeptide moiety so as to have e.g. at least one removed attachment group for the first non-polypeptide  
30 moiety and at least one introduced attachment group for the second non-polypeptide moiety or vice versa. For instance, the interferon  $\beta$  polypeptide comprises at least two (e.g. 2-5) removed attachment groups for the first non-polypeptide moiety and at least one (e.g. 1-5) introduced attachment groups for the second non-polypeptide moiety or vice versa. Of particular interest is a conjugate wherein the first non-polypeptide moiety is a polymer molecule such as PEG having

lysine as an attachment group, and the second non-polypeptide moiety is an N-linked sugar moiety.

More specifically, the conjugate of the invention may be one exhibiting interferon  $\beta$  activity and comprising at least one polymer molecule, preferably PEG, and at least one sugar moiety covalently attached to an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in

a) at least one introduced and/or at least one removed amino acid residue comprising an attachment group for the polymer molecule; and  
b) at least one introduced and/or at least one removed *in vivo* glycosylation site, in particular an N-glycosylation site,

provided that when the attachment group for the polymer molecule is a cysteine residue, and the sugar moiety is an N-linked sugar moiety, a cysteine residue is not inserted in such a manner that an N-glycosylation site is destroyed. WO 99/03887 suggests that a cysteine residue can be introduced into the natural N-glycosylation site of interferon  $\beta$ .

In a specific embodiment, the interferon  $\beta$  polypeptide comprises one of the following sets of mutations:

K19R+K45R+Q49N+Q51T+F111N+R113T+K123R;  
K19R+K45R+Q49N+Q51T+F111N+R113T; or  
K19R+K45R+Q49N+Q51T+ K123R.

Conjugate of the invention wherein the non-polypeptide moiety is a sugar moiety  
When the conjugate of the invention comprises at least one sugar moiety attached to an *in vivo* glycosylation site, in particular an N-glycosylation site, this is either the natural N-glycosylation site of wild-type human interferon  $\beta$  at position N80, i.e. defined by amino acid residues N80, E81, T82 and I83, or a new *in vivo* glycosylation site introduced into the interferon  $\beta$  polypeptide. The *in vivo* glycosylation site may be an O-glycosylation site, but is preferably an N-glycosylation site.

More specifically, in one aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in at least one introduced glycosylation site, the conjugate further comprising at least one un-PEGylated sugar moiety attached to an introduced glycosylation site.

5 In another aspect the invention relates to a conjugate exhibiting interferon  $\beta$  activity and comprising an interferon  $\beta$  polypeptide, the amino acid sequence of which differs from that of wild-type human interferon  $\beta$  in that a glycosylation site has been introduced or removed, provided that if only a glycosylation site is removed (and thus that no glycosylation site is introduced) the interferon  $\beta$  polypeptide does not comprise one or more of the following  
10 substitutions: N80C, E81C or T82C. The latter substitution is suggested in WO 99/03887.

For instance, an *in vivo* glycosylation site is introduced into a position of the parent interferon  $\beta$  molecule occupied by an amino acid residue exposed to the surface of the molecule, preferably with more than 25% of the side chain exposed to the solvent, in particular more than 50% exposed to the solvent (these positions are identified in the Methods section  
15 herein). The N-glycosylation site is introduced in such a way that the N-residue of said site is located in said position. Analogously, an O-glycosylation site is introduced so that the S or T residue making up such site is located in said position. Furthermore, in order to ensure efficient glycosylation it is preferred that the *in vivo* glycosylation site, in particular the N residue of the N-glycosylation site or the S or T residue of the O-glycosylation site, is located within the first  
20 141 amino acid residues of the interferon  $\beta$  polypeptide, more preferably within the first 116 amino acid residues. Still more preferably, the *in vivo* glycosylation site is introduced into a position wherein only one mutation is required to create the site (i.e. where any other amino acid residues required for creating a functional glycosylation site is already present in the molecule).

Substitutions that lead to introduction of an additional N-glycosylation site at  
25 positions exposed at the surface of the interferon  $\beta$  molecule and occupied by amino acid residues having more than 25% of the side chain exposed to the surface include:

S2N+N4S/T, L6S/T, L5N+G7S/T, F8N+Q10S/T, L9N+R11S/T, R11N, R11N+S13T,  
S12N+N14S/T, F15N+C17S/T, Q16N+Q18S/T, Q18N+L20S/T, K19N+L21S/T,  
W22N+L24S/T, Q23N+H25S/T, G26N+L28S/T, R27N+E29S/T, L28S+Y30S/T,  
30 Y30N+L32S/T, L32N+D34S/T, K33N+R35S/T, R35N+N37S/T, M36N+F38S/T, D39S/T,  
D39N+P41S/T, E42N+I44S/T, Q43N+K45S/T, K45N+L47S/T, Q46N+Q48S/T, L47N+Q49T/S,  
Q48N+F50S/T, Q49N+Q51S/T, Q51N+E53S/T, K52N+D54S/T, L57N+I59S/T, Q64N+I66S/T,  
A68N+F70S/T, R71N+D73S/T, Q72N, Q72N+S74T, D73N, D73N+S75T, S75N+T77S, S75N,  
S76N+G78S/T, E81N+I83S/T, T82N+V84S/T, E85N+L87S/T, L88S/T, A89N+V91S/T,  
35 Y92S/T, Y92N+Q94S/T, H93N+I95S/T, L98S/T, H97N+K99S/T, K99N+V101S/T,

5 T100N+L102S/T, E103N+K105S/T, E104N+L106S/T, K105N+E107S/T, E107N+E109S/T,  
 K108N+D110S/T, E109N+F111S/T, D110N+T112S, D110N, F111N+R113S/T,  
 R113N+K115S/T, G114N+L116S/T, K115N+M117S/T, L116N, L116N+S118T,  
 S119N+H212S/T, L120N+L122S/T, H121N+K123S/T, K123N+Y125S/T, R124N+Y126S/T,  
 G127N+I129S/T, R128N+L130S/T, L130N+Y132S/T, H131N+L133S/T, K134N+K136S/T,  
 10 A135N+E137S/T, K136N+Y138S/T, E137N, Y138N+H140S/T, H140N+A142S/T,  
 V148N+I150S/T, R152N+F154S/T, Y155N+I157S/T, L160S/T, R159N+T161S, R159N,  
 G162N+L164S/T, and Y163N+R165S/T.

Substitutions that lead to introduction of an additional N-glycosylation site at  
 positions exposed at the surface of the interferon  $\beta$  molecule having more than 50% of the side  
 15 chain exposed to the surface include: L6S/T, L5N+G7S/T, F8N+Q10S/T, L9N+R11S/T,  
 S12N+N14S/T, F15N+C17S/T, Q16N+Q18S/T, K19N+L21S/T, W22N+L24S/T,  
 Q23N+H25S/T, G26N+L28S/T, R27N+E29S/T, Y30N+L32S/T, K33N+R35S/T,  
 R35N+N37S/T, M36N+F38S/T, D39S/T, D39N+P41S/T, E42N+I44S/T, Q46N+Q48S/T,  
 Q48N+F50S/T, Q49N+Q51S/T, Q51N+E53S/T, K52N+D54S/T, L57N+I59S/T, R71N+D73S/T,  
 20 D73N, D73N+S75T, S75N+T77S, S75N, S76N+G78S/T, E81N+I83S/T, T82N+V84S/T,  
 E85N+L87S/T, A89N+V91S/T, Y92S/T, Y92N+Q94S/T, H93N+I95S/T, T100N+L102S/T,  
 E103N+K105S/T, E104N+L106S/T, E107N+E109S/T, K108N+D110S/T, D110N+T112S,  
 D110N, F111N+R113S/T, R113N+K115S/T, L116N, L116N+S118T, K123N+Y125S/T,  
 R124N+Y126S/T, G127N+I129S/T, H131N+L133S/T, K134N+K136S/T, A135N+E137S/T,  
 25 E137N, V148N+I150S/T, and Y155N+I157S/T.

Among the substitutions mentioned in the above lists, those are preferred that  
 have the N residue introduced among the 141 N-terminal amino acid residues, in particular  
 among the 116 N-terminal amino acid residues.

Substitutions that lead to introduction of an N-glycosylation site by only one  
 30 amino acid substitution include: L6S/T, R11N, D39S/T, Q72N, D73N, S75N, L88S/T, Y92S/T,  
 L98S/T, D110N, L116N, E137N, R159N and L160S/T. Among these, a substitution is preferred  
 that is selected from the group consisting of L6S/T, R11N, D39S/T, Q72N, D73N, S75N,  
 L88S/T, Y92S/T, L98S/T, D110N and L116N, more preferably from the group consisting of  
 L6S/T, D39S/T, D73N, S75N, L88S/T, D110N, L116N and E137N; and most preferably

5 selected from the group consisting of L6S/T, D39S/T, D73N, S75N, L88S/T, D110N and L116N.

One especially preferred interferon  $\beta$  polypeptide according to this aspect include at least one of the following substitutions:

10 S2N+N4T/S, L9N+R11T/S, R11N, S12N+N14T/S, F15N+C17S/T, Q16N+Q18T/S, K19N+L21T/S, Q23N+H25T/S, G26N+L28T/S, R27N+E29T/S, L28N+Y30T/S, D39T/S, K45N+L47T/S, Q46N+Q48T/S, Q48N+F50T/S, Q49N+Q51T/S, Q51N+E53T/S, R71N+D73T/S, Q72N, D73N, S75N, S76N+G78T/S, L88T/S, Y92T/S, N93N+I95T/S, L98T/S, E103N+K105T/S, E104N+L106T/S, E107N+E109T/S, K108N+D110T/S, D110N, F111N+R113T/S, or L116N, more preferably at least one of the following substitutions:  
15 S2N+N4T, L9N+R11T, 49N+Q51T or F111N+R113T or R71N+D73T, in particular 49N+Q51T or F111N+R113T or R71N+D73T. For instance, the interferon  $\beta$  polypeptide comprises one of the following sets of substitutions :

20 Q49N+Q51T+F111N+R113T ;  
Q49N+Q51T+R71N+D73T+ F111N+ R113T ;  
S2N+N4T+ F111N+R113T ;  
S2N+N4T+Q49N+Q51T ;  
S2N+N4T+Q49N+Q51T+F111N+R113T ;  
S2N+N4T+L9N+R11T+Q49N+Q51T ;  
S2N+N4T+L9N+R11T+F111N+R113T ;  
25 S2N+N4T+L9N+R11T+Q49N+Q51T+F111N+R113T ;  
L9N+R11T+Q49N+Q51T;  
L9N+R11T+Q49N+Q51T+F111N+R113T ; or  
L9N+R11T+F111N+R113T

It will be understood that in order to introduce a functional *in vivo* glycosylation  
30 site the amino acid residue inbetween the N-residue and the S/T residue is different from proline. Normally, the amino acid residue inbetween will be that occupying the relevant position in the amino acid sequence shown in SEQ ID NO 2. For instance, in the polypeptide comprising the substitutions Q49N+Q51S, position 50 is the position inbetween.

The interferon  $\beta$  polypeptide part of a conjugate of the invention may contain a  
35 single *in vivo* glycosylation site. However, in order to obtain efficient shielding of epitopes present on the surface of the parent polypeptide it is often desirable that the polypeptide comprises more than one *in vivo* glycosylation site, in particular 2-7 *in vivo* glycosylation sites, such as 2, 3, 4, 5, 6 or 7 *in vivo* glycosylation sites. Thus, the interferon  $\beta$  polypeptide may comprise one additional glycosylation site, or may comprise two, three, four, five, six, seven or

5 more introduced *in vivo* glycosylation sites, preferably introduced by one or more substitutions described in any of the above lists.

As indicated above, in addition to one or more introduced glycosylation sites, existing glycosylation sites may have been removed from the interferon  $\beta$  polypeptide. For instance, any of the above listed substitutions to introduce a glycosylation site may be combined  
10 with a substitution to remove the natural N-glycosylation site of human wild-type interferon  $\beta$ . For instance, the interferon  $\beta$  polypeptide may comprise a substitution of N80, e.g. one of the substitutions N80K/C/D/E, when a first non-polypeptide polypeptide is one having one of K, C, D, E as an attachment group. For instance, the interferon  $\beta$  polypeptide may comprise at least one of the following substitutions: S2N+N4T/S, L9N+R11T/S, R11N, S12N+N14T/S,  
15 F15N+C17S/T, Q16N+Q18T/S, K19N+L21T/S, Q23N+H25T/S, G26N+L28T/S, R27N+E29T/S, L28N+Y30T/S, D39T/S, K45N+L47T/S, Q46N+Q48T/S, Q48N+F50T/S, Q49N+Q51T/S, Q51N+E53T/S, R71N+D73T/S, Q72N, D73N, S75N, S76N+G78T/S, L88T/S, Y92T/S, N93N+I95T/S, L98T/S, E103N+K105T/S, E104N+L106T/S, E107N+E109T/S, K108N+D110T/S, D110N, F111N+R113T/S, or L116N in combination with N80K/C/D/E. More  
20 specifically, the interferon  $\beta$  polypeptide may comprise the substitution: Q49N+Q51T or F111N+R113T or R71N+D73T, in particular Q49N+Q51T+F111N+R113T or Q49N+Q51T+R71N+D73T+ F111N+ R113T, in combination with N80K/C/D/E.

Any of the glycosylated variants disclosed in the present section having introduced and/or removed at least one glycosylation site, such as the variant comprising the  
25 substitutions Q48N+F50T/S, Q48N+F50T/S+F111N+R113T/S, Q49N+Q51T/S, F111N+R113T/S, or Q49N+Q51T/S+F111N+R113T/S, may further be conjugated to a polymer molecule, such as PEG, or any other non-polypeptide moiety. For this purpose the conjugation may be achieved by use of attachment groups already present in the interferon  $\beta$  polypeptide or attachment groups may have been introduced and/or removed, in particular such that a total of 1-  
30 6, in particular 3-4 or 1, 2, 3, 4, 5, or 6 attachment groups are available for conjugation.

Preferably, in a conjugate of the invention wherein the interferon  $\beta$  polypeptide comprises two glycosylation sites, the number and molecular weight of the non-polypeptide moiety is chosen so as that the total molecular weight added by the non-polypeptide moiety is in the range of 20-40 kDa, in particular about 20 kDa or 30 kDa.



5 In particular, the glycosylated variant may be conjugated to a non-polypeptide moiety via a lysine attachment group, and one or more lysine residues of the parent polypeptide may have been removed, e.g. by any of the substitutions mentioned in the section entitled "Conjugate of the invention, wherein the non-polypeptide moiety is a molecule which has lysine as an attachment group", in particular the substitutions K19R+K45R+K123R. Alternatively or  
10 additionally, a lysine residue may have been introduced, e.g. by any of the substitutions mentioned in said section, in particular the substitution R71K. Accordingly, one specific conjugate of the invention is one, which comprises a glycosylated interferon  $\beta$  polypeptide comprising the mutations Q49N + Q51T + F111N + R113T + K19R + K45R + K123R or Q49N + Q51T + F111N + R113T + K19R + K45R + K123R + R71K further conjugated to PEG. The  
15 glycosylated polypeptide part of said conjugate is favourably produced in CHO cells and PEGylated subsequent to purification using e.g. SS-PEG, NPC-PEG, aldehyd-PEG, mPEG-SPA, mPEG-SCM, mPEG-BTC from Shearwater Polymers, Inc, SC-PEG from Enzon, Inc., tresylated mPEG as described in US 5,880,255, or oxycarbonyl-oxy-N-dicarboxyimide-PEG (US 5,122,614).

20 Alternatively, to PEGylation via a lysine group, the glycosylated conjugate according to this embodiment may be PEGylated via a cysteine group as described in the section entitled "Conjugate of the invention, wherein the non-polypeptide moiety is a molecule that has cysteine as an attachment group" (for this purpose the interferon  $\beta$  polypeptide may, e.g. comprising at least one of the mutations N80C, R71C and C17S), via an acid group as described  
25 in the section entitled "Conjugation of the invention wherein the non-polypeptide moiety binds to an acid group", or via any other suitable group.

#### Other conjugates of the invention

In addition to the introduction and/or removal of amino acid residues comprising an attachment group for the non-polypeptide moiety of choice (as described in any of the  
30 sections above entitled "Conjugate of the invention ....") the interferon  $\beta$  polypeptide part of the conjugate may contain further substitutions. A preferred example is a substitution of any of the residues, M1, C17, N80 or V101, e.g. one or more of the following substitutions: C17S; N80K/C/D/E; V101Y/W/F/H; a deletion of M1; or M1K. The substitution M1K is of particular interest when the interferon  $\beta$  polypeptide is expressed with a tag, e.g. a His-14tag, where such  
35 tag is to be removed by DAP (diaminopeptidase) subsequent to purification and/or conjugation.

5                   Non-polypeptide moiety of the conjugate of the invention

As indicated further above the non-polypeptide moiety of the conjugate of the invention is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a sugar moiety (by way of *in vivo* glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the polypeptide part of the conjugate, in particular reduced immunogenicity and/or increased functional *in vivo* half-life and/or increased serum half-life. The polypeptide part of the conjugate may be conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivating agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially. The choice of non-polypeptide moiety/ies, e.g. depends on the effect desired to be achieved by the conjugation. For instance, sugar moieties have been found particularly useful for reducing immunogenicity, whereas polymer molecules such as PEG are of particular use for increasing functional *in vivo* half-life and/or serum half-life. Using a polymer molecule as a first non-polypeptide moiety and a sugar moiety as a second non-polypeptide moiety may result in reduced immunogenicity and increased functional *in vivo* or serum half-life.

Methods of preparing a conjugate of the invention

In the following sections "Conjugation to a lipophilic compound", "Conjugation to a polymer molecule", "Conjugation to a sugar moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described.

Conjugation to a lipophilic compound

For conjugation to a lipophilic compound the following polypeptide groups may function as attachment groups: the N-terminal or C-terminal of the polypeptide, the hydroxy groups of the amino acid residues Ser, Thr or Tyr, the  $\epsilon$ -amino group of Lys, the SH group of Cys or the carboxyl group of Asp and Glu. The polypeptide and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other

multiple unsaturated compounds. The conjugation between the polypeptide and the lipophilic compound, optionally through a linker may be done according to methods known in the art, e.g. as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

#### Conjugation to a polymer molecule

The polymer molecule to be coupled to the polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or heteropolymer, typically with a molecular weight in the range of 300-100,000 Da, such as 300-20,000 Da, more preferably in the range of 500-10,000 Da, even more preferably in the range of 500-5000 Da.

Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH<sub>2</sub>) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer, which comprises one or more different coupling groups, such as, e.g., a hydroxyl group and an amine group.

Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-malic acid anhydride, dextran including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional *in vivo* half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

PEG is the preferred polymer molecule to be used, since it has only few reactive groups capable of cross-linking compared, e.g., to polysaccharides such as dextran, and the like. In particular, monofunctional PEG, e.g. monomethoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, the risk of cross-linking is eliminated, the resulting polypeptide conjugates are more homogeneous and the reaction of the polymer molecules with the polypeptide is easier to control.

5 To effect covalent attachment of the polymer molecule(s) to the polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups (examples of which include primary amino groups, hydrazide (HZ), thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA), succinimidyl propionate (SPA), succinimidyl carboxymethylate (SCM), benzotriazole carbonate (BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate (TRES)). Suitably activated polymer molecules are commercially available, e.g. from Shearwater Polymers, Inc., Huntsville, AL, USA. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference). Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g. SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG), BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in US 5,932,462 and US 5,643,575, both of which references are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: US 5,824,778, US 5,476,653, WO 97/32607, EP 229,108, EP 402,378, US 4,902,502, US 5,281,698, US 5,122,614, US 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, US 5,736,625, WO 98/05363, EP 809 996, US 5,629,384, WO 96/41813, WO 96/07670, US 5,473,034, US 5,516,673, EP 605 963, US 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

The conjugation of the polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): Harris and Zalipsky, eds., Poly(ethylene glycol) Chemistry and Biological Applications, AZC, Washington; R.F.

5 Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.;  
S.S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca  
Raton; G.T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic  
Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation  
10 chemistry to be used depends on the attachment group(s) of the interferon  $\beta$  polypeptide as well  
as the functional groups of the polymer (e.g. being amino, hydroxyl, carboxyl, aldehyde or sulfy-  
dryl). The PEGylation may be directed towards conjugation to all available attachment groups on  
the polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or  
may be directed towards specific attachment groups, e.g. the N-terminal amino group (US  
5,985,265). Furthermore, the conjugation may be achieved in one step or in a stepwise manner  
15 (e.g. as described in WO 99/55377).

It will be understood that the PEGylation is designed so as to produce the optimal  
molecule with respect to the number of PEG molecules attached, the size and form (e.g. whether  
they are linear or branched) of such molecules, and where in the polypeptide such molecules are  
attached. For instance, the molecular weight of the polymer to be used may be chosen on the basis  
20 of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to  
achieve a conjugate having a high molecular weight (e.g. to reduce renal clearance) it is usually  
desirable to conjugate as few high Mw polymer molecules as possible to obtain the desired  
molecular weight. When a high degree of epitope shielding is desirable this may be obtained by use  
of a sufficiently high number of low molecular weight polymer (e.g. with a molecular weight of  
25 about 5,000 Da) to effectively shield all or most epitopes of the polypeptide. For instance, 2-8, such  
as 3-6 such polymers may be used.

In connection with conjugation to only a single attachment group on the protein  
(as described in US 5,985,265), it may be advantageous that the polymer molecule, which may  
be linear or branched, has a high molecular weight, e.g. about 20 kDa.

30 Normally, the polymer conjugation is performed under conditions aiming at  
reacting all available polymer attachment groups with polymer molecules. Typically, the molar  
ratio of activated polymer molecules to polypeptide is 1000-1, in particular 200-1, preferably  
100-1, such as 10-1 or 5-1 in order to obtain optimal reaction. However, also equimolar ratios  
may be used.

5 It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; US 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378.

10 Subsequent to the conjugation residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules removed by a suitable method.

Covalent *in vitro* coupling of a carbohydrate moiety to amino acid residues of interferon  $\beta$  may be used to modify or increase the number or profile of carbohydrate substituents. Depending on the coupling mode used, the carbohydrate(s) may be attached to a) arginine and histidine (Lundblad and Noyes, Chemical Reagents for Protein Modification, CRC Press Inc. Boca Raton, FI), b) free carboxyl groups (e.g. of the C-terminal amino acid residue, asparagine or glutamine), c) free sulfhydryl groups such as that of cysteine, d) free hydroxyl groups such as those of serine, threonine, tyrosine or hydroxyproline, e) aromatic residues such as those of phenylalanine or tryptophan or f) the amide group of glutamine. These amino acid residues constitute examples of attachment groups for a carbohydrate moiety, which may be introduced and/or removed in the interferon  $\beta$  polypeptide. Suitable methods of *in vitro* coupling are described in WO 87/05330 and in Aplin etl al., CRC Crit Rev. Biochem., pp. 259-306, 1981. The *in vitro* coupling of carbohydrate moieties or PEG to protein- and peptide-bound Gln-residues can also be carried out by transglutaminases (TGases), e.g. as described by Sato et al., 1996 Biochemistry 35, 13072-13080 or in EP 725145

#### Coupling to a sugar moiety

In order to achieve *in vivo* glycosylation of an interferon  $\beta$  polypeptidethat has been modified by introduction of one or more glycosylation sites (see the section "Conjugates of the invention wherein the non-polypeptide moiety is a sugar moiety"), the nucleotide sequence encoding the polypeptide part of the conjugate must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect, mammalian animal cells, from transgenic plant cells or from transgenic animals. Furthermore, the glycosylation may be achieved in the human body when using a nucleotide sequence encoding the polypeptide part of a conjugate of the invention or a polypeptide of the

invention in gene therapy. In one embodiment the host cell is a mammalian cell, such as an CHO cell, BHK or HEK cell, e.g. HEK293, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *Saccharomyces cerevisiae*, *Pichia pastoris* or any other suitable glycosylating host, e.g. as described further below. Optionally, sugar moieties attached to the interferon  $\beta$  polypeptide by *in vivo* glycosylation are further modified by use of glycosyltransferases, e.g. using the glycoAdvance<sup>TM</sup> technology marketed by Neose, Horsham, PA, USA. Thereby, it is possible to, e.g., increase the sialylation of the glycosylated interferon  $\beta$  polypeptide following expression and *in vivo* glycosylation by CHO cells.

#### Coupling to an organic derivatizing agent

Covalent modification of the interferon  $\beta$  polypeptide may be performed by reacting (an) attachment group(s) of the polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with  $\alpha$ -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone,  $\alpha$ -bromo- $\beta$ -(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysiny and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysiny residues. Other suitable reagents for derivatizing  $\alpha$ -amino-containing residues include imidoesters such as methyl picolinimate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate. Arginy residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl or C-terminal amino acid residue) are

5 selectively modified by reaction with carbodiimides ( $R-N=C=N-R'$ ), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginy and glutaminy residues by reaction with ammonium ions.

#### Blocking of functional site

10 It has been reported that excessive polymer conjugation can lead to a loss of activity of the interferon  $\beta$  polypeptide to which the polymer is conjugated. This problem can be eliminated, e.g., by removal of attachment groups located at the functional site or by blocking the functional site prior to conjugation. These latter strategies constitute further embodiments of the invention (the first strategy being exemplified further above, e.g. by removal of lysine residues  
15 which may be located close to a functional site). More specifically, according to the second strategy the conjugation between the interferon  $\beta$  polypeptide and the non-polypeptide moiety is conducted under conditions where the functional site of the polypeptide is blocked by a helper molecule capable of binding to the functional site of the polypeptide. Preferably, the helper molecule is one, which specifically recognizes a functional site of the polypeptide, such as a  
20 receptor, in particular the type I interferon receptor. Alternatively, the helper molecule may be an antibody, in particular a monoclonal antibody recognizing the interferon  $\beta$  polypeptide. In particular, the helper molecule may be a neutralizing monoclonal antibody.

25 The polypeptide is allowed to interact with the helper molecule before effecting conjugation. This ensures that the functional site of the polypeptide is shielded or protected and consequently unavailable for derivatization by the non-polypeptide moiety such, as a polymer. Following its elution from the helper molecule, the conjugate between the non-polypeptide moiety and the polypeptide can be recovered with at least a partially preserved functional site.

30 The subsequent conjugation of the polypeptide having a blocked functional site to a polymer, a lipophilic compound, an organic derivatizing agent or any other compound is conducted in the normal way, e.g. as described in the sections above entitled "Conjugation to ....".

35 Irrespective of the nature of the helper molecule to be used to shield the functional site of the polypeptide from conjugation, it is desirable that the helper molecule is free from or comprises only a few attachment groups for the non-polypeptide moiety of choice in part(s) of the molecule, where the conjugation to such groups will hamper the desorption of the conjugated



5 polypeptide from the helper molecule. Hereby, selective conjugation to attachment groups present in non-shielded parts of the polypeptide can be obtained and it is possible to reuse the helper molecule for repeated cycles of conjugation. For instance, if the non-polypeptide moiety is a polymer molecule such as PEG, which has the epsilon amino group of a lysine or N-terminal amino acid residue as an attachment group, it is desirable that the helper molecule is substantially  
10 free from conjugatable epsilon amino groups, preferably free from any epsilon amino groups. Accordingly, in a preferred embodiment the helper molecule is a protein or peptide capable of binding to the functional site of the polypeptide, which protein or peptide is free from any conjugatable attachment groups for the non-polypeptide moiety of choice.

15 In a further embodiment, the helper molecule is first covalently linked to a solid phase such as column packing materials, for instance Sephadex or agarose beads, or a surface, e.g. reaction vessel. Subsequently, the polypeptide is loaded onto the column material carrying the helper molecule and conjugation carried out according to methods known in the art, e.g. as described in the sections above entitled "Conjugation to ....". This procedure allows the polypeptide conjugate to be separated from the helper molecule by elution. The polypeptide  
20 conjugate is eluted by conventional techniques under physico-chemical conditions that do not lead to a substantive degradation of the polypeptide conjugate. The fluid phase containing the polypeptide conjugate is separated from the solid phase to which the helper molecule remains covalently linked. The separation can be achieved in other ways: For instance, the helper molecule may be derivatised with a second molecule (e.g. biotin) that can be recognized by a  
25 specific binder (e.g. streptavidin). The specific binder may be linked to a solid phase thereby allowing the separation of the polypeptide conjugate from the helper molecule-second molecule complex through passage over a second helper-solid phase column which will retain, upon subsequent elution, the helper molecule-second molecule complex, but not the polypeptide conjugate. The polypeptide conjugate may be released from the helper molecule in any  
30 appropriate fashion. De-protection may be achieved by providing conditions in which the helper molecule dissociates from the functional site of the interferon  $\beta$  to which it is bound. For instance, a complex between an antibody to which a polymer is conjugated and an anti-idiotypic antibody can be dissociated by adjusting the pH to an acid or alkaline pH.

5                    Conjugation of a tagged interferon  $\beta$  polypeptide

In an alternative embodiment the interferon  $\beta$  polypeptide is expressed, as a fusion protein, with a tag, i.e. an amino acid sequence or peptide stretch made up of typically 1-30, such as 1-20 or 1-15 or 1-10 amino acid residues. Besides allowing for fast and easy purification, the tag is a convenient tool for achieving conjugation between the tagged polypeptide and the non-polypeptide moiety. In particular, the tag may be used for achieving conjugation in microtiter plates or other carriers, such as paramagnetic beads, to which the tagged polypeptide can be immobilised via the tag. The conjugation to the tagged polypeptide in, e.g., microtiter plates has the advantage that the tagged polypeptide can be immobilised in the microtiter plates directly from the culture broth (in principle without any purification) and subjected to conjugation. Thereby, the total number of process steps (from expression to conjugation) can be reduced. Furthermore, the tag may function as a spacer molecule ensuring an improved accessibility to the immobilised polypeptide to be conjugated. The conjugation using a tagged polypeptide may be to any of the non-polypeptide moieties disclosed herein, e.g. to a polymer molecule such as PEG.

20                    The identity of the specific tag to be used is not critical as long as the tag is capable of being expressed with the polypeptide and is capable of being immobilised on a suitable surface or carrier material. A number of suitable tags are commercially available, e.g. from Unizyme Laboratories, Denmark. For instance, the tag may be any of the following sequences:

25                    His-His-His-His-His-His  
Met-Lys-His-His-His-His-His  
Met-Lys-His-His-Ala-His-His-Gln-His-His  
Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln  
(vectors useful for providing such tags are available from Unizyme Laboratories, Denmark)

30                    or any of the following:

EQKLI SEEDL (a C-terminal tag described in Mol. Cell. Biol. 5:3610-16, 1985)  
DYKDDDDK (a C- or N-terminal tag)  
YPYDVPDYA

Antibodies against the above tags are commercially available, e.g. from ADI,  
35                    Aves Lab and Research Diagnostics.

A convenient method for using a tagged polypeptide for PEGylation is given in the Materials and Methods section below.

5 The subsequent cleavage of the tag from the polypeptide may be achieved by use of commercially available enzymes.

#### Polypeptides of the Invention

In further aspects the invention relates to generally novel interferon  $\beta$  polypeptides described herein that, as compared to human wildtype interferon  $\beta$  has at least one introduced and/or at least one removed attachment group for a non-polypeptide moiety . The novel polypeptides are important intermediate compounds for the preparation of a conjugate of the invention. In addition, the polypeptides themselves may have interesting properties.

Examples of such polypeptides include those that comprises an amino acid sequence which differs from that of wild-type human interferon  $\beta$  in that at least one amino acid residue selected from the group consisting of N4, F8, L9, Q10, R11, S13, L24, N25, G26, L28, E29, N37, F38, Q48, Q49, Q64, N65, I66, F67, A68, I69, F70, R71, Q72, D73, S74, S75, S76, T77, G78, W79, N80, E81, T82, I83, V84, L87, L88, A89, N90, V91, Y92, H93, Q94, D110, F111, T112, R113, R128, H140, T144, I145, R147, V148, L151, R152, F154, Y155, N158 and N166 is replaced with a different amino acid residue selected from the group consisting of K, R, D, E, C and N. The amino acid residues specified above are located in positions, which are exposed at the surface of human interferon  $\beta$  molecule as demonstrated by the solved 3D structure of human interferon  $\beta$ . By replacing one or more of these residue with either of K, R, D, E, C and N attachment group(s) for a non-polypeptide moiety, in particular a polymer attachment group or an amino acid residue susceptible to modification by a carbohydrate moiety, is/are introduced into human interferon  $\beta$ . The resulting modified human interferon  $\beta$  molecule is a suitable starting compound for the preparation of an interferon  $\beta$  conjugate having improved properties as compared to the unmodified human interferon  $\beta$  molecule.

In a further aspect the invention relates to an interferon  $\beta$  polypeptide comprising an amino acid sequence which differs from that of wild-type human interferon  $\beta$  in that at least one amino acid residue selected from the group consisting of N4, F8, L9, Q10, R11, S12, S13, L24, N25, G26, L28, E29, N37, F38, D39, Q48, Q49, Q64, N65, I66, F67, A68, I69, F70, R71, Q72, D73, S74, S75, S76, T77, G78, W79, N80, E81, T82, I83, V84, E85, L87, L88, A89, N90, V91, Y92, H93, Q94, D110, F111, T112, R113, R128, H140, T144, I145, R147, V148, L151, R152, F154, Y155, N158, G162, and N166 is replaced with a lysine residue, provided that the polypeptide is different from the one having the amino acid sequence of wild-type human

5 interferon  $\beta$  with the following substitutions: D54N+E85K+V91I+V101M and different from one which is a hybrid molecule between interferon  $\beta$  and interferon  $\alpha$  which as a consequence of being a hybrid has a lysine in position 39. The first of the disclaimed polypeptides is disclosed by Stewart et al, DNA Vol 6 no2 1987 p119-128 and was found to be inactive, the second is disclosed in US 4,769,233 and was constructed with the purpose of improving the biological  
10 activity of interferon  $\beta$ . None of the disclaimed polypeptides were made for or described as being suitable intermediates for the preparation of interferon  $\beta$  conjugates with reduced immunogenicity and/or prolonged functional *in vivo* half-life and/or serum half-life.

A still further example includes an interferon  $\beta$  polypeptide comprising an amino acid sequence which differs from that of SEQ ID NO 2 in one or more substitutions selected  
15 from the group consisting of N4K, F15K, Q16K, R27K, R35K, D39K, Q49K, E85K, A89K, E103K, E109K, R124K, E137K and R159K, provided that when the substitution is R27K the polypeptide is different from the one having the amino acid sequence of wild-type human interferon  $\beta$  with the following substitutions: R27K+E43K. The disclaimed polypeptide is disclosed by Stewart et al, DNA Vol 6 no2 1987 p119-128 and was found to have a low activity.  
20 The polypeptide was made in the course of a study of function-structure relationship and was not mentioned as a possible intermediate product for the preparation of improved interferon  $\beta$  conjugate molecules. For instance, the interferon  $\beta$  polypeptide comprises an amino acid sequence, which differs from that of SEQ ID NO 2 in that it comprises the substitution R27K in combination with at least one additional substitution that is different from E43K, or the  
25 substitution R35K in combination with at least one additional substitution provided that the polypeptide has an amino acid sequence which is different from the amino acid sequence of wild-type human interferon  $\beta$  modified with the following substitutions: G7E+S12N+C17Y+R35K. The disclaimed polypeptide is disclosed by Stewart et al, DNA Vol 6 no2 1987 p119-128 as having a retained antiproliferative activity on Daudi cells relative to their  
30 antiviral activity, but reduced overall activity as compared to wild type interferon  $\beta$ . The disclaimed polypeptide was not prepared with the purpose of reducing the immunogenicity and/or increasing the functional *in vivo* half-life and/or serum half-life, but was made in the course of a study of the structural functional relationship of interferon  $\beta$ .

5 The polypeptide of the invention may, in addition to any of the above specified substitutions, additionally comprise the substitution C17S and/or a deletion of M1 or the substitution M1K. Furthermore, the polypeptide of the invention may comprise an amino acid sequence, which further differs from that of SEQ ID NO 2 in the removal, preferably by substitution, of at least one lysine residue selected from the group consisting of K19, K33, K45,  
10 K52, K99, K105, K108, K115, K123, K134, and K136. The lysine residue(s) may be replaced with any other amino acid residue, but is preferably replaced by an arginine or a glutamine. In particular, the polypeptide of the invention may be one, wherein K45, K52 and/or K123 has/have been replaced with another amino acid residue, but preferably an arginine or a glutamine residue. Also, the polypeptide may be expressed with a tag, e.g. as described in the section further above  
15 entitled "Conjugation of a tagged interferon  $\beta$  polypeptide".

A still further example of an interferon  $\beta$  polypeptide of the invention includes one, that comprises an amino acid sequence which differs from that of wild-type human interferon  $\beta$  in that at least one lysine residue selected from the group consisting of K19, K33, K45, K52, K99, K105, K108, K115, K123, K134, and K136 has been replaced with any other  
20 amino acid residue, provided that the interferon  $\beta$  polypeptide is different from a hybrid between interferon  $\beta$  and interferon  $\alpha$ , which as a consequence of being a hybrid has a phenylalanine in position 45. Preferably, at least K19, K45, K52 and/or K123 is/are are replaced. While the lysine residue may be deleted in accordance with this aspect of the invention, it is preferred that it be replaced with any other amino acid residue, preferably an arginine or a glutamine. Normally, the  
25 polypeptide of the invention comprises an amino acid sequence which differs in 1-15 amino acid residues from the amino acid sequence shown in SEQ ID NO 2 as further discussed above. Examples of polypeptides of the invention are selected from the group consisting of those that comprises an amino acid sequence, which differs from that of SEQ ID NO 2 in at least the following substitutions:

30 R27K+R159K;  
R27K+K45R+R159K;  
R27K+Q49K+E85K+A89K;  
R27K+K45R+Q49K+E85K+A89K;  
R27K+D39K+Q49K+E85K+A89K;  
35 R27K+D39K+K45R+Q49K+E85K+A89K;  
N4K+R27K+D39K+Q49K+E85K+A89K;  
N4K+R27K+D39K+K45R+Q49K+E85K+A89K;

005280 " 082500 0964869 69584960

- 5 R27K+K123R+R159K;  
R27K+K45R+K123R+R159K;  
R27K+Q49K+E85K+A89K+K123R;  
R27K+K45R+Q49K+E85K+A89K+K123R;  
R27K+D39K+Q49K+E85K+A89K+K123R;  
10 R27K+D39K+K45R+Q49K+E85K+A89K+K123R;  
N4K+R27K+D39K+Q49K+E85K+A89K+K123R; and  
N4K+R27K+D39K+K45R+Q49K+E85K+A89K+K123R.

It will be understood that any of the polypeptides of the invention disclosed herein may be used to prepare a conjugate of the invention, i.e. be covalently coupled to any of the non-polypeptide moieties disclosed herein. In particular, when a polypeptide of the invention is expressed in a glycosylating microorganism the polypeptide may be provided in glycosylated form.

Methods of preparing an interferon  $\beta$  polypeptide for use in the invention

The polypeptide of the present invention or the polypeptide part of a conjugate of the invention, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and expressing the sequence in a suitable transformed or transfected host. However, polypeptides of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

The nucleotide sequence of the invention encoding an interferon  $\beta$  polypeptide may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent interferon  $\beta$ , e.g. with the amino acid sequence shown in SE Q ID NO 2, and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or deletion (i.e. removal or substitution) of the relevant amino acid residue(s).

The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with well-known methods, see, e.g., Mark et al., "Site-specific Mutagenesis of the Human Fibroblast Interferon Gene", Proc. Natl. Acad. Sci. USA, 81, pp. 5662-66 (1984); and US 4,588,585.

Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and

5 assembled by PCR, ligation or ligation chain reaction (LCR). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the interferon  $\beta$  polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the interferon  $\beta$  in the  
10 desired transformed host cell.

It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding a polypeptide variant described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression  
15 control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be  
20 considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their  
25 fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

The recombinant vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced  
30 into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral  
35 DNA. A number of suitable expression vectors for expression in the host cells mentioned herein

5 are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, CA, USA) and pCI-neo (Stratagene, La Jola, CA, USA). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as  
10 plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages. Useful expression vectors for yeast cells include the 2 $\mu$  plasmid and derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in (Okkels,  
15 Ann. New York Acad. Sci. 782, 202-207, 1996) and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen).

Other vectors for use in this invention include those that allow the nucleotide  
20 sequence encoding the polypeptide variant to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrofolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase  
25 ("GS") amplification (see, e.g., US 5,122,464 and EP 338,841).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2 $\mu$  replication genes REP 1-3  
30 and origin of replication.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin,



5 tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niaD, sC.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide.

10 Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1 $\alpha$  (EF-1 $\alpha$ ) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50).

In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide of interest. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

30 Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence.

Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast  $\alpha$ -mating system, the yeast triose phosphate isomerase (TPI) promoter,

5 promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter and the inducible GAL promoter.

Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* 10  $\alpha$ -amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator.

Examples of suitable control sequences for use in bacterial host cells include promoters of the *lac* system, the *trp* system, the *TAC* or *TRC* system and the major promoter regions of phage lambda.

15 The nucleotide sequence of the invention encoding an interferon  $\beta$  polypeptide, whether prepared by site-directed mutagenesis, synthesis or other methods, may or may not also include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide. The 20 signal peptide may be homologous (e.g. be that normally associated with human interferon  $\beta$ ) or heterologous (i.e. originating from another source than human interferon  $\beta$ ) to the polypeptide or may be homologous or heterologous to the host cell, i.e. be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli*, or eukaryotic, 25 e.g. derived from a mammalian, or insect or yeast cell.

The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide, the protein to be expressed (whether it is an intracellular or extracellular protein) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an 30 *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral  $\alpha$ -amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic 35 hormone precursor, (cf. US 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid

5 UDPglucosyltransferase (egt) (Murphy et al., Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of human interferon  $\beta$  apparent from the examples hereinafter or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be  
10 the  $\alpha$ -factor signal peptide from *S. cerevisiae*. (cf. US 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., Nature 289, 1981, pp. 643-646), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), and the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137).

15 Any suitable host may be used to produce the interferon  $\beta$  polypeptide, including bacteria, fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis*, *Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector  
20 into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne,  
25 1987, *Journal of Bacteriology* 169: 5771-5278).

Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae*, *A. niger*, or *A. nidulans*, *Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of  
30 *Aspergillus* host cells are described in EP 238 023 and US 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156 and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York;

5 Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920.

Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae*, *Schizosaccharomyces*, *Kluyveromyces*, *Pichia*, such as *P. pastoris* or *P. methanolica*, *Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Methods for transforming yeast cells with  
10 heterologous DNA and producing heterologous polypeptides therefrom are disclosed by Clontech Laboratories, Inc, Palo Alto, CA, USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit), and by Reeves *et al.*, *FEMS Microbiology Letters* 99 (1992) 193-198, Manivasakam and Schiestl, *Nucleic Acids Research*, 1993, Vol. 21, No. 18, pp. 4414-4415 and Ganeva *et al.*, *FEMS Microbiology Letters* 121 (1994) 159-164.

15 Examples of suitable insect host cells include a *Lepidoptera* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusia ni* cells (High Five) (US 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen.

Examples of suitable mammalian host cells include Chinese hamster ovary (CHO)  
20 cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection,  
25 Rockville, Maryland. Also, the mammalian cell, such as a CHO cell, may be modified to express sialyltransferase, e.g. 1,6-sialyltransferase, e.g. as described in US 5,047,335, in order to provide improved glycosylation of the interferon  $\beta$  polypeptide.

Methods for introducing exogenous DNA into mammalian host cells include  
calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection,  
30 liposome-mediated transfection, viral vectors and the transfection methods described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000 and Roche Diagnostics Corporation, Indianapolis, USA using FuGENE 6. These methods are well known in the art and e.g. described by Ausbel *et al.* (eds.), 1996, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods,  
35 e.g. as disclosed in *(Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel*

5 Jenkins, 1999, Human Press Inc, Totowa, New Jersey, USA and Harrison MA and Rae IF, General Techniques of Cell Culture, Cambridge University Press 1997).

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale  
10 fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to  
15 published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional  
20 procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric  
25 focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989). Specific methods for purifying polypeptides exhibiting interferon  $\beta$  activity are disclosed in US 4,289,689, US 4,359,389, US 4,172,071, US 4,551,271, US 5,244,655, US 4,485,017, US 4,257,938 and US 4,541,952. A specific purification method is based on  
30 immunoaffinity purification (see, e.g., Okamura et al., "Human Fibroblastoid Interferon: Immunosorbent Column Chromatography And N-Terminal Amino Acid Sequence", *Biochem.*, 19, pp. 3831-35 (1980)). Furthermore, purification may be based on the use of IFNAR 1 and/or IFNAR 2, in particular IFNAR 2.

The biological activity of the interferon  $\beta$  polypeptides can be assayed by any  
35 suitable method known in the art. Such assays include antibody neutralization of antiviral

activity, induction of protein kinase, oligoadenylate 2,5-A synthetase or phosphodiesterase activities, as described in EP 41313 B1. Such assays also include immunomodulatory assays (see, e.g., US 4,753,795), growth inhibition assays, and measurement of binding to cells that express interferon receptors. Specific assays for determining the biological activity of polypeptides or conjugates of the invention are disclosed in the Materials and Methods section hereinafter.

#### CELL CULTURE OF THE INVENTION

In a further aspect the invention relates to a cell culture comprising a) a host cell transformed with a nucleotide sequence encoding a polypeptide exhibiting interferon  $\beta$  activity, and b) a culture medium comprising said polypeptide produced by expression of said nucleotide sequence in a concentration of at least 800,000 IU/ml of medium, preferably in a concentration in the range of 800,000-3,500,000 IU/ml medium. While the polypeptide exhibiting interferon  $\alpha$  activity may be a wild-type interferon  $\beta$ , e.g. human interferon  $\beta$  or a variant thereof (e.g. interferon  $\beta$  1a or 1b) the polypeptide is preferably an interferon  $\beta$  polypeptide as described herein.

In a still further aspect the invention relates to a method of producing an interferon  $\beta$  polypeptide as described herein, the method comprising:

(a) culturing a cell expressing an interferon  $\beta$  polypeptide variant in a culture medium, such that the concentration of the interferon  $\beta$  polypeptide variant in the medium is at least 800,000 IU/ml medium, in particular in the range of between 800,000 and 3,500,000 IU/ml medium; and

(b) recovering the interferon  $\beta$  polypeptide.

#### OTHER METHODS OF THE INVENTION

In a still further aspect the invention relates to a method reducing immunogenicity and/or of increasing functional *in vivo* half-life and/or serum half-life of an interferon  $\beta$  polypeptide, which method comprises introducing an amino acid residue constituting an attachment group for a first non-polypeptide moiety into a position exposed at the surface of the protein that does not contain such group and/or removing an amino acid residue constituting an

5 attachment group for a first non-polypeptide moiety and subjecting the resulting modified polypeptide to conjugation with the first non-polypeptide moiety.

Preferably, the amino acid residue to be introduced and/or removed is as defined in the present application. The non-polypeptide moiety is normally selected from the group consisting of a polymer molecule, a sugar moiety, a lipophilic group and an organic derivatizing agent.

10 In a still further aspect the invention relates to a method for preparing a conjugate of the invention, wherein the interferon  $\beta$  polypeptide is reacted with the non-polypeptide moiety to which it is to be conjugated under conditions conducive for the conjugation to take place, and the conjugate is recovered.

15 Pharmaceutical composition and uses of a conjugate of the invention

The interferon  $\beta$  polypeptide or the conjugate of the invention is administered at a dose approximately paralleling that employed in therapy with human interferon  $\beta$  such as Avonex™, Rebif® and Betaseron®, or a higher dose. The exact dose to be administered depends on the circumstances. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide, conjugate or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide or conjugate or composition is administered alone or in conjunction with other therapeutic agents, the serum half-life of the compositions, and the general health of the patient.

25 The polypeptide or conjugate of the invention can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, lithium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may be present as a crystalline and/or amorphous structure.

30 The polypeptide or conjugate of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art.

The polypeptide or conjugate of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described in US

5 5,183,746, Remington's Pharmaceutical Sciences by E.W.Martin, 18<sup>th</sup> edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

10 The pharmaceutical composition of the polypeptide or conjugate of the invention may be formulated in a variety of forms, including liquid, gel, lyophilized, pulmonary dispersion, or any other suitable form, e.g. as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

15 The pharmaceutical composition containing the polypeptide or conjugate of the invention may be administered orally, intravenously, intracerebrally, intramuscularly, intraperitoneally, intradermally, subcutaneously, intranasally, intrapulmonary, by inhalation, or in any other acceptable manner, e.g. using PowderJect or ProLease technology. The preferred mode of administration will depend upon the particular indication being treated and will be apparent to one of skill in the art.

#### Parenterals

20 An example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form. In the former case, the composition must be thawed prior to use. The latter form is often used to enhance the stability of the active compound contained in the composition under a wider variety of storage conditions, as it is recognized by those skilled in the art that lyophilized preparations are generally more stable than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution.

30 In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic detergents, antioxidants and/or other miscellaneous additives.

35



5 Buffering agents help to maintain the pH in the range which approximates physiological conditions. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium gluconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar

5 alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol,  $\alpha$ -monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilize the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

The active ingredient may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacrylate) microcapsules, in colloidal drug delivery systems (for example liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

Parenteral formulations to be used for *in vivo* administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

### Sustained release preparations

Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the polypeptide or conjugate, the matrices having a suitable form such as a film or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)), polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up to or over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated polypeptides remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

### Pulmonary delivery

Conjugate formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the conjugate dissolved in water at a concentration of, e.g., about 0.01 to 25 mg of conjugate per mL of solution, preferably about 0.1 to 10 mg/mL. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure), and/or human serum albumin ranging in concentration from 0.1 to 10 mg/ml.

Examples of buffers which may be used are sodium acetate, citrate and glycine. Preferably, the buffer will have a composition and molarity suitable to adjust the solution to a pH in the range of 3 to 9. Generally, buffer molarities of from 1 mM to 50 mM are suitable for this purpose. Examples of sugars which can be utilized are lactose, maltose, mannitol, sorbitol, trehalose, and xylose, usually in amounts ranging from 1% to 10% by weight of the formulation.

5 The nebulizer formulation may also contain a surfactant to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol. Various conventional surfactants can be employed, such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbitan fatty acid esters. Amounts will generally range between 0.001% and 4% by weight of the formulation. An especially preferred surfactant for  
10 purposes of this invention is polyoxyethylene sorbitan monooleate.

Specific formulations and methods of generating suitable dispersions of liquid particles of the invention are described in WO 9420069, US 5915378, US 5960792, US 5957124, US 5934272, US 5915378, US 5855564, US 5826570 and US 5522385 which are hereby incorporated by reference.

15 Three specific examples of commercially available nebulizers suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Mo., the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado, and the AERx pulmonary drug delivery system manufactured by Aradigm Corporation, Hayward, California.

20 Conjugate formulations for use with a metered dose inhaler device will generally comprise a finely divided powder. This powder may be produced by lyophilizing and then milling a liquid conjugate formulation and may also contain a stabilizer such as human serum albumin (HSA). Typically, more than 0.5% (w/w) HSA is added. Additionally, one or more sugars or sugar alcohols may be added to the preparation if necessary. Examples include lactose  
25 maltose, mannitol, sorbitol, sorbitose, trehalose, xylitol, and xylose. The amount added to the formulation can range from about 0.01 to 200% (w/w), preferably from approximately 1 to 50%, of the conjugate present. Such formulations are then lyophilized and milled to the desired particle size.

30 The properly sized particles are then suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant. This mixture is then loaded into  
35 the delivery device. An example of a commercially available metered dose inhaler suitable for

5 use in the present invention is the Ventolin metered dose inhaler, manufactured by Glaxo Inc.,  
Research Triangle Park, N.C.

Such conjugate formulations for powder inhalers will comprise a finely divided  
dry powder containing conjugate and may also include a bulking agent, such as lactose, sorbitol,  
sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, e.g.,  
10 50% to 90% by weight of the formulation. The particles of the powder shall have aerodynamic  
properties in the lung corresponding to particles with a density of about 1 g/cm<sup>2</sup> having a median  
diameter less than 10 micrometers, preferably between 0.5 and 5 micrometers, most preferably of  
between 1.5 and 3.5 micrometers.

An example of a powder inhaler suitable for use in accordance with the teachings  
15 herein is the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Mass.

The powders for these devices may be generated and/or delivered by methods  
disclosed in US 5997848, US 5993783, US 5985248, US 5976574, US 5922354, US 5785049  
and US 5654007 which are hereby incorporated by reference.

The pharmaceutical composition containing the conjugate of the invention may be  
20 administered by a wide range of mechanical devices designed for pulmonary delivery of  
therapeutic products, including but limited to nebulizers, metered dose inhalers, and powder  
inhalers, all of which are familiar to those of skill in the art.

Some specific examples of commercially available devices suitable for the  
practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St.  
25 Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products,  
Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research  
Triangle Park, North Carolina; the Spinhaler powder inhaler, manufactured by Fisons Corp.,  
Bedford, Massachusetts; the "standing cloud" device of Inhale Therapeutic Systems, Inc., San  
Carlos, California; the AIR inhaler manufactured by Alkermes, Cambridge, Massachusetts; and  
30 the AERx pulmonary drug delivery system manufactured by Aradigm Corporation, Hayward,  
California.

The pharmaceutical composition of the invention may be administered in  
conjunction with other therapeutic agents. These agents may be incorporated as part of the same  
pharmaceutical composition or may be administered separately from the polypeptide or  
35 conjugate of the invention, either concurrently or in accordance with any other acceptable

5 treatment schedule. In addition, the polypeptide, conjugate or pharmaceutical composition of the invention may be used as an adjunct to other therapies.

Accordingly, this invention provides compositions and methods for treating most types of viral infections, cancers or tumors (e.g. breast carcinoma, non-small cell lung cancer) or tumour angiogenesis, Chrohn's disease, ulcerative colitis, Guillain-Barré syndrome, glioma,  
10 idiopathic pulmonary fibrosis, abnormal cell growth, or for immunomodulation in any suitable animal, preferably mammal, and in particular human. In particular the polypeptide, conjugate or composition of the invention may be used for the treatment of multiple sclerosis (MS), such as any of the generally recognized four types of MS (benign, relapsing remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS)) and for  
15 monosymptomatic MS), hepatitis, or a herpes infection (the latter treatment optionally being combined with a treatment with IL-10).

In a further aspect the invention relates to a method of treating a mammal having circulating antibodies against interferon  $\beta$  1a, such as Avonex<sup>TM</sup> or Rebif®, or 1b, such as Betaseron®, which method comprises administering a compound which has the bioactivity of  
20 interferon  $\beta$  and which has a reduced or no reaction with said antibodies. The compound is administered in an effective amount. The compound is preferably a conjugate as described herein and the mammal is preferably a human being. The mammals to be treated may suffer from any of the diseases listed above for which interferon  $\beta$  is a useful treatment. In particular, this aspect of the invention is of interest for the treatment of multiple sclerosis (any of the types listed above)  
25 or cancer. Furthermore, the invention relates to a method of making a pharmaceutical product for use in treatment of mammals having circulating antibodies against interferon  $\beta$  1a, such as Avonex<sup>TM</sup> or Rebif®, or 1b, such as Betaseron®, wherein a compound which has the bioactivity of interferon  $\beta$  and which does not react with such is formulated into an injectable or otherwise suitable formulation. The term "circulating antibodies" is intended to indicate antibodies, in  
30 particular neutralizing antibodies, formed in a mammal in response to having been treated with any of the commercially available interferon  $\beta$  preparations (Rebif®, Betaseron®, Avonex<sup>TM</sup>).

In a further aspect the invention relates to a method of treating a patient in need of treatment with a pharmaceutical composition with at least some of the therapeutically beneficial properties of interferon  $\beta$  comprising administering a composition comprising a compound with  
35 at least part of the therapeutically beneficial activity of interferon  $\beta$ , said treatment having

5 reduced or removed adverse psychological effects as compared to treatment with interferon  $\beta$ , wherein said compound is a non-naturally occurring conjugate of a polypeptide with interferon  $\beta$  activity and a non-polypeptide moiety, in particular a conjugate according to the present invention.

10 In a still further aspect the invention relates to a pharmaceutical composition for the treatment of a patient in need of treatment with a compound having at least part of the therapeutically beneficial properties of interferon  $\beta$ , said composition comprising a compound which is a non-naturally occurring conjugate of interferon  $\beta$  and a non-polypeptide moiety, said treatment further giving rise to fewer adverse psychological effects than treatment with interferon  $\beta$ . The conjugate is preferably a conjugate of the invention.

15 Also contemplated is use of a nucleotide sequence encoding a polypeptide of the invention in gene therapy applications. In particular, it may be of interest to use a nucleotide sequence encoding a polypeptide as described in the section above entitled "Glycosylated Polypeptides of the Invention modified to incorporate additional glycosylation sites". The glycosylation of the polypeptides is thus achieved during the course of the gene therapy, i.e. after expression of the nucleotide sequence in the human body.

20 Gene therapy applications contemplated include treatment of those diseases in which the polypeptide is expected to provide an effective therapy due to its antiviral activity, e.g., viral diseases, including hepatitis such as hepatitis C, and particularly HPV, or other infectious diseases that are responsive to interferon  $\beta$  or infectious agents sensitive to interferon  $\beta$ . Furthermore, the conjugate or polypeptide of the invention may be used in the treatment of chronic inflammatory demyelinating polyradiculoneuropathy, and of severe necrotising cutaneous lesions. Also, gene therapy in connection with the treatment of any MS type is contemplated. Similarly, this invention contemplates gene therapy applications for immunomodulation, as well as in the treatment of those diseases in which interferon  $\beta$  is expected to provide an effective therapy due to its antiproliferative activity, e.g., tumors and cancers, or other conditions characterized by undesired cell proliferation, such as restenosis. A further description of such gene therapy is provided in WO 95/25170.

35 Local delivery of interferon  $\beta$  using gene therapy may provide the therapeutic agent to the target area while avoiding potential toxicity problems associated with non-specific administration.

5 Both *in vitro* and *in vivo* gene therapy methodologies are contemplated.

Several methods for transferring potentially therapeutic genes to defined cell populations are known. For further reference see, e.g., Mulligan, "The Basic Science Of Gene Therapy", Science, 260, pp. 926-31 (1993). These methods include:

10 Direct gene transfer, e.g., as disclosed by Wolff et al., "Direct Gene transfer Into Mouse Muscle *In vivo*", Science 247, pp. 1465-68 (1990);

Liposome-mediated DNA transfer, e.g., as disclosed by Caplen et al., "Liposome-mediated CFTR Gene Transfer to the Nasal Epithelium Of Patients With Cystic Fibrosis" Nature Med., 3, pp. 39-46 (1995); Crystal, "The Gene As A Drug", Nature Med., 1, pp.- 15-17 (1995); Gao and Huang, "A Novel Cationic Liposome Reagent For Efficient Transfection of Mammalian  
15 Cells", Biochem.Biophys Res. Comm., 179, pp. 280-85 (1991);

Retrovirus-mediated DNA transfer, e.g., as disclosed by Kay et al., "In vivo Gene Therapy of Hemophilia B: Sustained Partial Correction In Factor IX-Deficient Dogs", Science, 262, pp. 117-19 (1993); Anderson, "Human Gene Therapy", Science, 256, pp.808-13(1992);

20 DNA Virus-mediated DNA transfer. Such DNA viruses include adenoviruses (preferably Ad-2 or Ad-5 based vectors), herpes viruses (preferably herpes simplex virus based vectors), and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably AAV-2 based vectors). See, e.g., Ali et al., "The Use Of DNA Viruses as Vectors for Gene Therapy", Gene Therapy, 1, pp. 367-84 (1994); US 4,797,368, and US 5,139,941.

25 The invention is further described in the following examples. The examples should not, in any manner, be understood as limiting the generality of the present specification and claims.

## MATERIALS AND METHODS

### Materials

30 HeLa cells – (available from American Type Culture Collection (ATCC)  
ISRE-Luc (Stratagene, La Jolla USA)  
pCDNA 3.1/hygro (Invitrogen, Carlsbad USA)  
pGL3 basic vector (Promega)  
Human genomic DNA (CloneTech, USA)



- 5 DMEM medium: Dulbecco's Modified Eagle Media (DMEM), 10% fetal bovine serum (available from Life Technologies A/S, Copenhagen, Denmark)

## ASSAYS

### Interferon Assay

It has previously been published that interferon  $\beta$  interacts with and activates

- 10 Interferon type I receptors on HeLa cells. Consequently, transcription is activated at promoters containing an Interferon Stimulated Response Element (ISRE). It is thus possible to screen for agonists of interferon receptors by use of an ISRE coupled luciferase reporter gene (ISRE-luc) placed in HeLa cells.

### Primary Assay

15 HeLa cells are co-transfected with ISRE-Luc and pCDNA 3.1/hygro and foci (cell clones) are created by selection in DMEM media containing Hygromycin B. Cell clones are screened for luciferase activity in the presence or absence of interferon  $\beta$ . Those clones showing the highest ratio of stimulated to unstimulated luciferase activity are used in further assays.

- 20 To screen muteins, 15,000 cells/well are seeded in 96 well culture plates and incubated overnight in DMEM media. The next day muteins as well as a known standard are added to the cells in various concentrations. The plates are incubated for 6 hours at 37 C in a 5% CO<sub>2</sub> air atmosphere LucLite substrate (Packard Bioscience, Groningen The Netherlands ) is subsequently added to each well. Plates are sealed and luminescence measured on a TopCount  
25 luminometer (Packard) in SPC (single photon counting) mode. Each individual plate contains wells incubated with interferon  $\beta$  as a stimulated control and other wells containing normal media as an unstimulated control. The ratio between stimulated and unstimulated luciferase activity serves as an internal standard for both mutein activity and experiment-to-experiment variation.

### Secondary Assay

- 30 Currently, there are 18 non-allelic interferon  $\alpha$  genes and one interferon  $\beta$  gene. These proteins exhibit overlapping activities and thus it is critical to ensure that muteins retain the selectivity and specificity of interferon  $\beta$ .

- 35 The  $\beta$ -R1 gene is activated by interferon  $\beta$  but not by other interferons. The transcription of  $\beta$ -R1 thus serves as a second marker of interferon  $\beta$  activation and is used to

5 ensure that muteins retain interferon  $\beta$  activity. A 300 bp promoter fragment of  $\beta$ -R1 shown to drive interferon sensitive transcription (Rani. M.R. et al (1996) *JBC* 271 22878-22884 ) was isolated by PCR from human genomic DNA and inserted into the pGL3 basic vector (Promega). The resulting  $\beta$ -R1:luciferase gene is used in assays similar to the primary assay described above. In astrocytoma cells, the resulting  $\beta$ -R1:luciferase gene has been described to show 250  
10 fold higher sensitivity to interferon  $\beta$  than to interferon  $\alpha$  (Rani et al. *op cit*).

#### ELISA assay

The concentration of IFN- $\beta$  is quantitated by use of a commercial sandwich immunoassay (PBL Biomedical Laboratories, New Brunswick, NJ, USA). The kit is based on an ELISA with monoclonal mouse anti-IFN- $\beta$  antibodies for catching and detection of IFN- $\beta$  in test  
15 samples. The detecting antibody is conjugated to biotin.

Tests samples and recombinant human IFN- $\beta$  standard are added in 0.1 mL in concentrations from 10-0.25 ng/mL to microtiter plates, precoated with catching antibody. The plates are incubated at RT for 1 hr. Samples and standard are diluted in kit dilution buffer.

The plates are washed in the kit buffer and incubated with the biotinylated  
20 detecting antibody in 0.1 mL for 1 hr at RT. After another wash the streptavidin-horseradishperoxidase conjugate is added in 0.1 mL and incubated for 1 hr at RT.

The reaction is visualised by addition of 0.1 mL Tetramethylbenzidine (TMB) substrate chromogen. The plates are incubated for 15 minutes in the dark at RT and the reaction is stopped by addition of stop solution. The absorbance is read at 450nm using an ELISA reader.

#### Receptor binding assay

The receptor binding capability of a polypeptide or conjugate of the invention can be determined using the assay described in WO 95/25170 entitled "Analysis Of IFN- $\beta$ (Phe<sub>101</sub>) For Receptor Binding"(which is based on Daudi or A549 cells). Soluble domains of IFNAR1 and IFNAR2 can be obtained essentially as described by Arduini et al, Protein Science, 1999,  
25 vol. 8, 1867-1877 or as described in Example 9 herein.

Alternatively, the receptor binding capability is determined using a crosslinking agent such as disuccinimidyl suberate (DSS) available from Pierce, Rockford, IL, USA as follows:

The polypeptide or conjugate is incubated with soluble IFNAR-2 receptor in the  
35 presence or absence of DSS in accordance with the manufacturer's instructions. Samples are

5 separated by SDS-PAGE, and a western blot using anti-interferon  $\beta$  or anti-IFNAR2 antibodies is performed. The presence of a functional interferon  $\beta$  polypeptide/conjugate: receptor interaction is apparent by an increase in the molecular size of receptor and interferon  $\beta$  in the presence of DSS.

10 Furthermore, a crosslinking assay using a polypeptide or conjugate of the invention and both receptor subunits (IFNAR-1 and IFNAR-2) can establish Interferon receptor 1 binding ability. In this connection it has been published that IFNAR-1 binds only after an interferon  $\beta$ : IFNAR-2 complex is formed (Mogensen et al., Journal of Interferon and Cytokine Research, 19:1069-1098, 1999).

In vitro immunogenicity tests of interferon  $\beta$  conjugates

15 Reduced immunogenicity of a conjugate or polypeptide of the invention is determined by use of an ELISA method measuring the immunoreactivity of the conjugate or polypeptide relative to a reference molecule or preparation. The reference molecule or preparation is normally a recombinant human interferon  $\beta$  preparation such as Avonex, Rebif or Betaseron, or another recombinant human interferon  $\beta$  preparation produced by a method  
20 equivalent to the way these products are made. The ELISA method is based on antibodies from patients treated with one of these recombinant interferon  $\beta$  preparations. The immunogenicity is considered to be reduced when the conjugate or polypeptide of the invention has a statistically significant lower response in the assay than the reference molecule or preparation.

25 Another method of determining immunogenicity is by use of sera from patients treated with interferon beta (i.e. any commercial interferon  $\beta$  product) in an analogous manner to that described by Ross et al. J. Clin Invest. 95, 1974-78, 1995. In the antiviral neutralisation bioassay reduced immunogenicity results in reduced inhibition of a conjugate of the invention by patient sera compared to a wt IFN-beta reference molecule. Furthermore, in the biochemical IFN binding assay a less immunogenic conjugate is expected to bind to patient IgG to a lesser extent  
30 than reference IFN-beta molecules.

For the neutralisation assay, the reference and conjugate molecules are added in a concentration that produces approximately 80% virus protection in the antiviral neutralisation bioassay. The IFN- $\beta$  proteins are mixed with patient sera in various dilutions (starting at 1:20).

5                    Antiviral activity

The antiviral bioassay is performed using A549 cells (CCL 185, American tissue culture collection) and Encephalomyocarditis (EMC) virus (VR-129B, American tissue culture collection).

10                    The cells are seeded in 96 well tissue culture plates at a concentration of 10,000 cells/well and incubated at 37°C in a 5% CO<sub>2</sub> air atmosphere. A polypeptide or conjugate of the invention is added in concentrations from 100-0.0001 IU/mL in a total of 100µl DMEM medium containing fetal calf serum and antibiotics.

15                    After 24 hours the medium is removed and 0.1 mL fresh medium containing EMC virus is added to each well. The EMC virus is added in a concentration that causes 100% cell death in IFN-β free cell cultures after 24 hours.

20                    After another 24 hrs, the antiviral effect of the polypeptide or conjugate is measured using the WST-1 assay. 0.01 mL WST-1 (WST-1 cell proliferation agent, Roche Diagnostics GmbH, Mannheim, Germany) is added to 0.1 mL culture and incubated for ½-2 hours at 37°C in a 5% CO<sub>2</sub> air atmosphere. The cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells results in the formation of formazan that is quantified by measuring the absorbance at 450 nm.

25                    Neutralisation of activity in Interferon Stimulated Response Element (ISRE) assay

The interferon β neutralising effect of anti-interferon β sera are analysed using the ISRE-Luciferase activity assay.

30                    Sera from interferon β treated patients or from immunised animals are used. Sera are added either in a fixed concentration (dilution 1:20-1:500 (pt sera) or 20-600 ng/mL (animal sera)) or in five-fold serial dilutions of sera starting at 1/20 (pt sera) or 600 ng/mL (animal sera). Interferon β is added either in five fold-dilutions starting at 25.000 IU/mL or in a fixed concentration (0.1-10 IU/mL) in a total volume of 80µl DMEM medium + 10% FCS. The sera are incubated for 1 hr. at 37°C with IFN-β.

35                    The samples are then transferred to 96 well tissue culture plates containing HeLa cells transfected with ISRE-Luc grown from 24 hrs before (15,000 cells/well) in DMEM media. The cultures are incubated for 6 hours at 37°C in a 5% CO<sub>2</sub> air atmosphere. LucLite substrate (Packard Bioscience, Groningen, The Netherlands) is subsequently added to each well. Plates

are sealed and luminescence measured on a TopCount luminometer (Packard) in SPC (single photon counting) mode.

When interferon  $\beta$  samples are titrated in the presence of a fixed amount of serum, the neutralising effect was defined as fold inhibition (FI) quantified as  $EC50(w. serum)/EC50 (w/o serum)$ . The reduction of antibody neutralisation of interferon  $\beta$  variant proteins is defined as

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$$(1 - \frac{FI \text{ variant}}{FI \text{ wt}}) \times 100\%$$

Biological half-life measurement of a PEG - interferon  $\beta$  conjugate

Measurement of biological half-life can be carried out in a number of ways described in the literature. One method is described by Munafo et al (European Journal of Neurology 1998, vol 5 No2 p 187-193), who used an ELISA method to detect serum levels of interferon  $\beta$  after subcutaneous and intramuscular administration of interferon  $\beta$ .

The rapid decrease of interferon  $\beta$  serum concentrations after i.v. administration has made it important to evaluate biological responses to interferon  $\beta$  treatment. However it is contemplated that the conjugates of the present invention will have prolonged serum half lives also after i.v. administration making it possible to measure by e.g. an ELISA method or by the primary screening assay.

Different pharmacodynamic markers (e.g. serum neuplerin and beta2 microglobulin) have also been studied (Clin Drug Invest (1999) 18(1):27-34). These can equally well be used to evaluate prolonged biological effect. These experiments may also be carried out in suitable animal species, e.g. rats.

Assays to assess the biological effects of interferon  $\beta$  such as antiviral, antiproliferative and immunomodulatory effects (as described in e.g. Annals of Neurology 1995 vol 37 No 1 p 7-15) can be used together with the primary and secondary screening assays described herein to evaluate the biological efficacy of the conjugate in comparison to wild type interferon  $\beta$ .

Finally an animal model such as the commonly used experimental autoimmune encephalomyelitis (EAE) model can be used to establish efficacy of a conjugate or polypeptide of the invention. In the EAE model immunization with myelin or myelin derived proteins elicits a disease mimicking the majority of the inflammatory and neurologic features of multiple

5 sclerosis in humans. EAE has been used in mice, rats, rabbits, and marmosets {Cannella, Hoban, et al. 1998 ID: 695} {Zaprianova, Deleva, et al. 1997 ID: 699} {Hassouna, Galeano, et al. 1983 ID: 731} {Genain & Hauser 1997 ID: 703}. Other models include Theiler's murine encephalomyelitis virus (TMEV) model {Murray, McGavern, et al. 1998 ID: 694}. will be used to establish efficacy of the interferon  $\beta$  conjugate.

10 PEGylation in microtiter plates of a tagged polypeptide with interferon  $\beta$  activity  
The method comprises

Expressing the interferon  $\beta$  polypeptide with a suitable tag, e.g. any of the tags exemplified in the general description above.

Transferring culture broth to one or more wells in a microtiter plate capable of  
15 immobilising the tagged polypeptide. When the tag is His-His-His-His-His (Casey et al, J. Immunol. Meth., 179, 105 (1995)), a Ni-NTA HisSorb microtiter plate commercially available from QiaGen can be used.

After allowing for immobilising the tagged polypeptide to the microtiter plate, the wells are washed in a buffer suitable for binding and subsequent PEGylation.

20 Incubating the wells with the activated PEG of choice. As an example, M-SPA-5000 from Shearwater Polymers is used. The molar ratio of activated PEG to polypeptide has to be optimised, but will typically be greater than 10:1 more typically greater than 100:1.

After a suitable reaction time at ambient temperature, typically around 1 hour, the reaction is stopped by removal of the activated PEG solution. The conjugated protein is eluted  
25 from the plate by incubation with a suitable buffer. Suitable elution buffers may contain Imidazole, excess NTA or another chelating compound.

The conjugated protein is assayed for biological activity and immunogenicity as appropriate.

This tag may optionally be cleaved off using a method known in the art, e.g. using  
30 diaminopeptidase and the Gln in pos -1 will be converted to pyroglutamyl with GCT (glutamylcyclotransferase) and finally cleaved off with PGAP (pyro-glutamyl-aminopeptidase) giving the native protein. The process involves several steps of metal chelate affinity chromatography. Alternatively, the tagged polypeptide may be conjugated.

5                   PEGylation of a receptor-bound interferon  $\beta$  polypeptide

In order to optimize PEGylation of an interferon  $\beta$  polypeptide in a manner excluding PEGylation of lysines involved in receptor recognition, the following method has been developed: The soluble domains of IFNAR1 and IFNAR2 are obtained essentially as described in Arduini et al, Protein Science (1999), vol 8: 1867-1877 or as described in Example 9.

10                   A ternary complex consisting of an interferon  $\beta$  polypeptide, a soluble domain of IFNAR1 and a soluble domain of IFNAR2 in a 1:1:1 stoichiometry is formed in a PBS buffer at pH 7-9. The concentration of Interferon  $\beta$  polypeptide is approximately 20 ug/ml or 1 uM and the receptors are present at equimolar concentration.

M-SPA-5000 from Shearwater Polymers, Inc is added at 3 different concentration  
15 levels corresponding to 5, 20 or 100 molar excess of interferon  $\beta$  polypeptide. The reaction time is 30 min at RT. After the 30 min reaction period, the pH of the reaction mixture is adjusted to pH 2.0 and the reaction mixture is applied to a Vydac C18 column and eluted with an acetonitrile gradient essentially as described (Utsumi et al, J. Biochem., vol 101, 1199-1208, (1987). Alternatively and more elegantly, an isopropanol gradient can be used.

20                   Fractions are analyzed using the primary screening assay described herein and active PEGylated interferon- $\beta$  polypeptide obtained by this method stored at  $-80^{\circ}\text{C}$  in PBS, pH 7 containing 1 mg/ml HSA.

Alternatively, to the procedure described above a soluble domain of IFNAR2 is used as the only receptor component to form a binary complex. Furthermore, IFNAR2 may be  
25 immobilized on a suitable resin (e.g. Epoxy activated Sepharose 6B) according to the manufactures instructions prior to forming the binary complex. After PEGylation, the PEGylated Interferon- $\beta$  is eluted with a 0.1 M Glycin, pH 2 buffer and activity measured as described after pH adjustment to neutral.

30                   Accessible Surface Area (ASA)

The computer program Access (B. Lee and F.M.Richards, J. Mol.Biol. 55: 379-400 (1971)) version 2 (Copyright (c) 1983 Yale University) are used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4Å and defines the Accessible Surface Area (ASA) as the area formed by the centre of the probe. Prior to this calculation all water molecules and all hydrogen atoms are removed from  
35 the coordinate set, as are other atoms not directly related to the protein. Alternative programs are

5 available for computing ASA, e.g. the program WhatIf G.Vriend, J. Mol. Graph. (1990) 8, 52-56, electronically available at the WWW interface on <http://swift.embl-heidelberg.de/servers2/> (R.Rodriguez *et.al.* CABIOS (1998) 14, 523-528.) using the option *Accessibility* to calculate the accessible molecular surface.

#### Fractional ASA of side chain

10 The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended ALA-x-ALA tripeptide. See Hubbard, Campbell & Thornton (1991) J.Mol.Biol.220,507-530. For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table indicates the

15 100% ASA standard for the side chain:

Ala	69.23 Å <sup>2</sup>
Arg	200.35 Å <sup>2</sup>
Asn	106.25 Å <sup>2</sup>
Asp	102.06 Å <sup>2</sup>
Cys	96.69 Å <sup>2</sup>
Gln	140.58 Å <sup>2</sup>
Glu	134.61 Å <sup>2</sup>
Gly	32.28 Å <sup>2</sup>
His	147.00 Å <sup>2</sup>
Ile	137.91 Å <sup>2</sup>
Leu	140.76 Å <sup>2</sup>
Lys	162.50 Å <sup>2</sup>
Met	156.08 Å <sup>2</sup>
Phe	163.90 Å <sup>2</sup>
Pro	119.65 Å <sup>2</sup>
Ser	78.16 Å <sup>2</sup>
Thr	101.67 Å <sup>2</sup>
Trp	210.89 Å <sup>2</sup>
Tyr	176.61 Å <sup>2</sup>
Val	114.14 Å <sup>2</sup>

#### Determining surface exposed amino acid residues

The three-dimensional crystal structure of human interferon beta at 2.2 Å resolution (Karpusas *et al.* Proc. Nat. Acad. Sci. USA (1997) 94:11813-11818 is available from

20 the Protein Data Bank (PDB) (Bernstein *et.al.* J. Mol. Biol. (1977) 112 pp. 535) and



electronically available via The Research Collaboratory for Structural Bioinformatics PDB at <http://www.pdb.org/> under accession code 1AU1. This crystal structure contain two independent molecules of human interferon beta in this example the A molecule is used.

Surface exposure:

Using the WhatIf program as described above the following residues were found to have zero surface accessibility for their side chain atoms (for Gly the accessibility of the CA atom is used): G7, N14, C17, L21, I44, A55, A56, T58, I59, M62, L63, L98, L122, Y125, I129, L133, A142, W143, V146, I150, N153, I157, L160, T161, and L164.

Fractional surface exposure

For further analysis it was necessary to remodel the side chains of residues R71, R113, K115, L116, M117 due to steric clashes. The remodelling was done using Modeler 98, MSI INC. Performing fractional ASA calculations using the Access computer program on the remodelled interferon beta molecule (only including the amino acid residues and excluding the N-linked sugar moiety) resulted in the following residues having more than 25% of their side chain exposed to the surface: S2, N4, L5, F8, L9, R11, S12, F15, Q16 Q18, K19, W22, Q23, G26, R27, L28, E29, Y30, L32, K33, R35, M36, N37, D39, E42, K45, Q46, L47, Q48, Q49, Q51, K52, Q64, A68, R71, Q72, D73, S75, S76, G78, N80, E81, T82, E85, N86, A89, Y92, H93, N96, H97, K99, T100, E103, E104, K105, E107, K108, E109, D110, F111, R113, G114, K115, L116, S119, L120, H121, K123, R124, G127, R128, L130, H131, K134, A135, K136, E137, Y138, S139, H140, V148, R152, Y155, N158, G162, Y163, R165, and N166. and the following residues have more than 50% of their side chain exposed to the surface: N4, L5, F8, S12, F15, Q16, K19, W22, G26, R27, E29, Y30, K33, R35, N37, D39, E42, Q46, Q48, Q49, Q51, K52, R71, D73, S75, G78, N80, E81, T82, E85, N86, A89, Y92, H93, K99, T100, E103, E104, E107, K108, D110, F111, L116, K123, R124, G127, H131, K134, E137, V148, Y155, R165, and N166.

**EXAMPLES**

EXAMPLE 1. DESIGN OF AN EXPRESSION CASSETTE FOR EXPRESSION OF INTERFERON  $\beta$  IN MAMMALIAN AND INSECT CELLS

The DNA sequence, GenBank accession number M28622 (shown in SEQ ID NO 1), encompassing a full length cDNA encoding human interferon  $\beta$  with its native signal peptide, was modified in order to facilitate high expression in mammalian cells. First the ATG start codon

005230" 65584950

5 context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987  
Aug 20;196(4):947-50), such that there is a perfect match to the consensus sequence upstream of  
the ATG start codon. Secondly the codons of the native human interferon  $\beta$  was modified by  
making a bias in the codon usage towards the codons frequently used in highly expressed human  
genes. Subsequently, certain nucleotides in the sequence were substituted with others in order to  
10 introduce recognition sites for DNA restriction endonucleases (this allows for easier  
modification of the DNA sequence later). Primers were designed such that the gene could be  
synthesised:

CBProFpr1  
5'GGCTAGCGTTTAAACTTAAGCTTCGCCACCATGACCAACAAGTGCCTGCTC  
15 CAGATCGCCCTGCTCCTGT-3', SEQ ID 3,  
CBProFpr2  
5'ACAACCTGCTCGGCTTCCTGCAGAGGAGTTCGAACTTCCAGTGCCAGAAGC  
TCCTGTGGCAGCTGAACGG-3', SEQ ID 4,  
CBProFpr3  
20 5'GAACTTCGACATCCCCGAGGAAATCAAGCAGCTGCAGCAGTTCCAGAAGG  
AGGACGCCGCTCTGACCATC-3', SEQ ID 5,  
CBProFpr4  
5'TTCCGCCAGGACTCCAGCTCCACCGGTTGGAACGAGACCATCGTGGAGAAC  
CTGCTGGCCAACGTGTACC-3', SEQ ID 6,  
25 CBProFpr5  
5'AGGAGAAGCTGGAGAAGGAGGACTTCACCCGCGGCAAGCTGATGAGCTCC  
CTGCACCTGAAGCGCTACTA-3', SEQ ID 7,  
CBProFpr6  
5'GGAGTACAGCCACTGCGCCTGGACCATCGTACGCGTGGAGATCCTGCGCAA  
30 CTTCTACTTCATCAACCGC-3', SEQ ID 8,  
CBProFpr9  
5'CACCACACTGGACTAGTGGATCCTTATCAGTTGCGCAGGTAGCCGGTCAGG  
CGGTTGATGAAGTAGAAGT-3', SEQ ID 9,  
CBProFpr10  
35 5'AGGCGCAGTGGCTGTACTCCTTGGCCTTCAGGTAGTGCAGGATGCGGCCAT  
AGTAGCGCTTCAGGTGCAG-3', SEQ ID 10,  
CBProFpr11  
5'CTCCTTCTCCAGCTTCTCCTCCAGCACGGTCTTCAGGTGGTTGATCTGGTGG  
TACACGTTGGCCAGCAGG-3', SEQ ID 11,  
40 CBProFpr12  
5'GAGCTGGAGTCCTGGCGGAAGATGGCGAAGATGTTCTGCAGCATCTCGTAG  
ATGGTCAGAGCGGCGTCCT-3', SEQ ID 12,  
CBProFpr13  
45 5'CCTCGGGGATGTCGAAGTTCATCCTGTCCTTCAGGCAGTACTCCAGGCGCC  
CGTTCAGCTGCCACAGGAG-3', SEQ ID 13,

5 CBProFpr14

5'CAGGAAGCCGAGCAGGTTGTAGCTCATCGATAGGGCCGTGGTGCTGAAGC  
ACAGGAGCAGGGCGATCTGG-3', SEQ ID 14,

The primers were assembled to the synthetic gene by one step PCR using  
Platinum *Pfx*- polymerase kit (Life Technologies) and standard three step PCR cycling  
10 parameters. The assembled gene was amplified by PCR using the same conditions.

A cDNA encoding a N-terminal extended form of human interferon  $\beta$  was  
synthesised using the same PCR conditions as described above but with the primers CBProFpr1  
and -14 substituted with the primers:

15 CBProFpr7

5'CTGCTCCAGATCGCCCTGCTCCTGTGCTTCAGCACCACGGCCCTATCGATGA  
AGCACCAGCACCAGCATC-3', SEQ ID 15,

CBProFpr8

5'CACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCA  
20 AGCTGGCTAGCGTTTAAAC-3', SEQ ID 16,

CBProFpr15

5'CAGGAAGCCGAGCAGGTTGTAGCTCATCTGTTGGTGTTGATGTTGGTGCTG  
ATGCTGGTGCTGGTGCTTC-3', SEQ ID 17,

CBProFpr16

25 5'AGCAGGGCGATCTGGAGCAGGCACTTGTTGGTCATGGTGGCGAAGCTTAAG  
TTTAAACGCTAGCCAGCTT-3', SEQ ID 18,

in order to incorporate a purification TAG in the interferon  $\beta$  molecule.

The synthesised genes were cloned into pcDNA3.1/Hygro (Invitrogen) between  
the *HindIII* site at the 5' end and the *BamHI* at the 3', resulting in pCBProF1 and pCBProF2.

30 The synthetic intron from pCI-Neo (Promega) was amplified using standard PCR  
conditions as described above and the primers:

CBProFpr37 5'-CCGTCAGATCCTAGGCTAGCTTATTGCGGTAGTTTATCAC-3', SEQ ID  
19,

CBProFpr38 5'-GAGCTCGGTACCAAGCTTTTAAGAGCTGTAAT-3', SEQ ID 20,

35 resulting in a 332 bp PCR fragment which was cut with *NheI* and *HindIII* and inserted in the  
5'UTR of the plasmids pCBProF1 and pCBProF2 resulting in pCBProF4 and pCBProF5.

Codons for individual amino acids were changed by amplifying relevant regions  
of the coding region by PCR in such a way that the PCR introduced changes in the sequence can  
be introduced in the expression plasmids by classical cloning techniques. E.g. the primers:

40 Lys45arg-5'primer (*NarI*/*KasI*):

5'GCTGAACGGGCGCCTGGAGTACTGCCTGAAGGACAGGATGAACTTCGACATCCCC  
GAGGAAATCCGCCAGCTGCAGC-3', SEQ ID 21,

Lys45mut-3'primer (BsiWI): 5'TCTCCACGCGTACGATGGTCCAGGCGCAGTGGCTG-3',  
SEQ ID 22,  
were used to introduce a K45R substitution in the PCR-fragment spanning the region from  
position 1055 to 1243 in pCBProF1. Both the PCR fragment and pCBProF1 was cut with NarI  
and BsiWI which are both unique. The PCR fragment and the vector backbone of pCBProF1 are  
purified and ligated resulting in substitution of the Lys45 codon AAG with the Arg codon CGC  
in pCBProF1.

Furthermore, SOE (sequence overhang extension) PCR was used for introduction  
of amino acid substitutions. In the SOE-PCR both the N-terminal part and the C-terminal part of  
the INFB molecule were first amplified in individual primary PCRs.

For these primary PCRs the central complementary primers were synthesised such  
that the codon(s) for the amino acid(s) to be substituted is/are changed to the desired codon(s).  
The terminal primers were standard primers defining the N- and C-terminal of the INFB  
molecule respectively. Further the terminal primers provided a restriction enzyme site enabling  
subsequent cloning of the full-length PCR product. Thus, the central (nonsense) primer and the  
N-terminal (sense) primer were used to amplify the N-terminal part of the INFB coding region in  
one of the primary PCRs and equivalently for the C-terminal part. Once amplified the N- and C-  
terminal parts are assembled into the full-length product in a secondary PCR and cloned into a  
modified version of pCDNA3.1/Hygro as described above. For instance, the following primers  
were used to introduce the mutations for the substitutions F111N and R113T:

CBProFprimer9(Sense):  
CACCACACTGGACTAGTGGATCCTTATCAGTTGCGCAGGTAGCCGGTCAGGC  
GGTTG ATG AAGTAGAAGT (SEQ ID NO 23),

CBProFprimer231(Antisense):  
CATCAGCTTGCCGGTGGTGTGTCCTCCTTC (SEQ ID NO 24),

CBProFprimer230 (Sense):  
GAAGGAGGACAACACCACCGCAAGCTGATG (SEQ ID NO 25),

CBProFprimer42 (Antisense):  
CACACTGGACTAGTAAGCTTTTATCAGTTGCGCAGGTAGC (SEQ ID NO 26),

Furthermore, in cases where the introduced mutation(s) were sufficiently close to  
a unique restriction endo-nuclease site in the expression plasmid variant genes were constructed

5 using construction procedure encompassing a single PCR step and a subsequent cloning. For instance, the substitution K19R was introduced by use of the PCR primer:

CBProFpr58:

GAGGAGTTCGAACTTCCAGTGCCAGCGCCTCCTGTGGCAGCTGAACG (SEQ ID

NO 27), and CBProFprimer9:

10 The PCR product was subsequently cloned using the restriction endo-nuclease sites *Bsi*WI and *Bst*BI.

## EXAMPLE 2. EXPRESSION OF HUMAN INTERFERON $\beta$ IN A BACULOVIRUS/INSECT CELL SYSTEM

15 In order to express the synthetic gene, encoding human interferon  $\beta$  harboured in pCBProF1 (described in example 1) in the baculovirus/insect cell system the gene was excised with *Nhe*I and *Xho*I and ligated into the transfer vector pBlueBac 4.5, which is included in the MaxBac 2.0 Transfection kit obtained from Invitrogen (San Diego, USA). All methods used for generation of recombinant baculovirus and expression in insect cells are described in the  
20 "MaxBac 2.0 Transfection and Expression Manual" included in the kit.

In brief, together with linearized AcMNPV DNA (Bac-N-Blue DNA) pBlueBac 4.5-interferon  $\beta$  CBProF1) was transfected into SF9 cells. 3 days post-transfection the transfection supernatant was harvested and a plaque assay with appropriate viral dilutions was prepared. Blue distinct plaques were visible after 7 days and 6 individual plaques were collected  
25 for propagation in a 6-well plate. After 5 days 2 ml virus supernatant (P-1 stock) was harvested from each well. 0.75 ml was taken out from the P1 stocks and viral genomic DNA was isolated. The viral genomic DNA's were analysed in PCR reactions with forward/reverse primers in order to be able to select the recombinant baculoviruses among the six P-1 stocks. A small aliquot from the recombinant P-1 stock was tested in a human interferon  $\beta$  specific ELISA (available  
30 from PBL Biomedical Laboratories) in order to ensure that recombinant human interferon  $\beta$  was present in the supernatant.

For further propagation of chosen recombinant baculovirus  $6 \times 10^6$  SF9 cells were seeded in a T-80 culture flask and infected with 200  $\mu$ l of the P-1 stock. After 5 days the supernatant (P-2 stock) was harvested and 2 ml of the P-2 stock was used to infect a 100 ml

suspension culture ( $1 \times 10^6$  SF9 cells/ml) in a 500 ml Erlenmeyer flask (Corning). After 5 days the supernatant (P-3 stock) was harvested and the virus titer was determined by plaque assay.

In order to produce human interferon  $\beta$  for purification  $1 \times 10^9$  SF9 cells were harvested from a backup suspension culture. In a 50 ml screw-cap tube the SF9 cells were infected with recombinant baculovirus from the P-3 stock (MOI = 2) in a period of 15 minutes.

Hereafter the cells were spun down and washed one time in serum-free medium (Sf-900 II SFM, Gibco BRL) and transferred to a 2800 ml Triple Baffle Fernbach Flask (Bellco) containing 1 l serum-free medium. 3 days post-infection the medium supernatant was harvested and the recombinant human interferon  $\beta$  was purified.

#### Purification of interferon $\beta$ molecules

The fermentation broth is concentrated and/or pH adjusted to approximately 4.5 after dilution to suitable ionic strength. Suitable is intended to mean that the ionic strength is so low that interferon  $\beta$  will bind to a Mono S cation exchange column (Pharmacia) equilibrated in 4 mM acetic acid pH 4.5 (buffer A). After application, the column is washed with 3 column volumes buffer A and interferon  $\beta$  is eluted with a linear gradient from buffer A to buffer A including 1 M NaCl. Alternatively purification can be obtained as described for Interferon  $\alpha$  (Analytical Biochemistry 247, 434-440 (1997) using a TSK-gel SP-5PW column (Toso Haas)).

Alternatively His tagged interferon  $\beta$  can be purified using IMAC (Immobilized Metal Affinity Chromatography) in accordance with well known methods, e.g., as described by UniZyme Laboratories, Denmark.

Another purification method makes use of monoclonal or polyclonal antibodies. Interferon  $\beta$  fermentation broth is adjusted to pH 7 and 0.5 M NaCl and applied to a column with immobilized monoclonal antibody to recombinant human interferon  $\beta$ . The column is equilibrated with e.g. 10 mM Tris, 0.5 M NaCl, pH 7 (Buffer B) prior to application. After application the column is washed with 3 column volumes Buffer B and eluted with a suitable buffer at low pH (e.g. pH 2-3).

Alternatively, if the interferon  $\beta$  is tagged with e.g. the c-Myc peptide (EQKLI SEEDL), monoclonal antibodies raised against the c-Myc peptide, can be used in a similar fashion. Immobilization of antibody to the column is achieved using e.g. CNBr-Sepharose (Pharmacia) according to the manufacturers instructions.

5 A combination of Cation exchange chromatography, IMAC and/or antibody chromatography may be applied if necessary to obtain relevant purity for further experiments.

Purity, identity, quantity and activity of eluted fractions from the abovementioned columns can be determined using a combination of methods known by the person skilled in the art. These may include one or more of the following assays and methods or other relevant  
10 methods known by the person skilled in the art: the primary and secondary assays described above, ELISA methods, SDS-PAGE, western blotting, IEF, HPLC, amino acid sequencing, mass spectrometry and amino acid analysis.

Following purification, the modified interferon  $\beta$  polypeptide may be subjected to conjugation to a polymer molecule such as M-SPA-5000 from Shearwater Polymers according to  
15 the manufacturer's instructions. Preferably, the receptor recognition site of the purified modified interferon  $\beta$  polypeptide is blocked prior to conjugation as described in the Materials and Methods section herein.

### EXAMPLE 3. EXPRESSION OF HUMAN INTERFERON $\beta$ IN HEK293 CELLS

In order to express the synthetic gene, encoding human interferon  $\beta$ , harboured by  
20 pCBProF1 (described in example 1), in HEK293 cells (ATCC Cat. No. CRL-1573) the gene was PCR-amplified with the two primers PBR 7 (5'-CGCGGATCCATATGACCAACAAGTGCCTG-3') (SEQ ID NO 28) and PBR 2 (5'-CGCGGATCCTTATCAGTTGCGCAG-3') (SEQ ID NO 29) and cloned into the BamHI site of pcDNA3.1 (-) (Invitrogen, USA) in correct orientation, giving the plasmid pPR9.

25 For transfection of the HEK293 cell line a T-25 culture flask was seeded to 50% confluency in DMEM medium (Life Technologies, USA) containing 10% FBS and incubated over night. By usage of FuGENE 6 Transfection Reagent (Roche, USA) pPR9 was transfected into the cells: To 95  $\mu$ l serum-free DMEM medium was added 5  $\mu$ l FuGENE 6 and 1.7  $\mu$ l (2  $\mu$ g) pPR9 and incubated at room temperature for 20 minutes. The transfection complex was then  
30 added drop-wise to the cells and the culture flask was returned to the incubator. Next day the cells were trypsinized and seeded into a T-80 culture flask in DMEM medium containing 10% FBS and 500  $\mu$ g Geneticin (Life Technologies) per ml.

At confluency it was confirmed, by usage of a human interferon  $\beta$  specific ELISA, that the primary transfection-pool was expressing the wished protein and the cells were

5 sub-cloned by limited dilution. In this way a high-producing HEK293 clone was identified expressing human interferon  $\beta$ .

#### EXAMPLE 4. HIGH LEVEL EXPRESSION OF INTERFERON $\beta$ IN CHO CELLS

10 The cell line CHO K1 [p22]-E4 (ATCC # CCL-61) stably expressing human interferon  $\beta$  was passed 1:10 from a confluent culture and propagated as adherent cells in T-25 flasks in serum containing medium (MEM $\alpha$  w/ ribonucleotides and deoxyribonucleotides (Gibco/BRL Cat # 32571), 10% FCS (Gibco/BRL Cat # 10091), penicillin and streptomycin (Gibco/BRL Cat # 15140-114) until confluence. The media was then changed to serum free media (RenCyte CHO; MediCult Cat.# 22600140) for 24 hours before including 5 mM Sodium Butyrate (Merck Cat # 8.17500) during a medium change. The cells were then allowed to express interferon  $\beta$  for 48 hours prior to harvest of the medium. The interferon  $\beta$  concentration in the duplicate cultures were determined to be 854,797 IU/ml (with lower and upper 95% confidence interval at 711,134 IU/ml and 1,032,012 IU/ml) respectively).

20 In a separate set of experiments, the cell line CHO K1 [p22]-E4 stably expressing human interferon  $\beta$  was passed 1:10 from a confluent culture and propagated as adherent cells in serum containing medium (MEM $\alpha$  w/ ribonucleotides and deoxyribonucleotides (Gibco/BRL Cat # 32571), 10% FCS (Gibco/BRL Cat # 10091), penicillin and streptomycin (Gibco/BRL Cat # 15140-114) until confluence in a 10 layer cell factory (NUNC #165250). The media was then changed to serum free media; DMEM/F12 (Gibco/BRL # 11039-021) with the addition of 1:100 ITS-A (Gibco/BRL # 51300-044) and 1:500 EX-CYTE VLE (Serological Proteins Inc. # 81-129-1) and 1:100 penicillin and streptomycin (Gibco/BRL Cat # 15140-114) for 48 hours before changing the medium with the further addition of 5 mM butyrate (Merck Cat # 8.17500). The cells were then allowed to express interferon  $\beta$  for 48 hours prior to harvest of the medium. The interferon  $\beta$  concentration was determined to be 824,791 IU/ml (with lower and upper 95% confidence interval at 610,956 IU/ml and 1,099,722 IU/ml) respectively).

30 It is contemplated that interferon  $\beta$  polypeptides of the invention may be produced in equally high yields in the same manner as any of those described above.



5 EXAMPLE 5. CONSTRUCTION AND EXPRESSION OF INTERFERON  $\beta$  VARIANT  
WITH ONE INTRODUCED GLYCOSYLATION SITE

In order to insert an extra N-linked glycosylation site at position 111 in hINF- $\beta$ , the synthetic gene (*hinf- $\beta$* ) encoding hINF- $\beta$  (described in example 1) was altered by site-directed PCR mutagenesis. Using BIO-X-ACT (Bioline, UK) and the plasmid PF050 [*hinf-*

10  *$\beta$* /pcDNA3.1(-)Hygro/Intron (a derivative of pcDNA3.1(-)Hygro (Invitrogen, USA) in which a chimeric intron obtained from pCI-neo (Promega, USA) had been inserted between the BamHI and NheI sites in the MCS of the vector] as template, two PCR reactions were performed with two overlapping primer-sets [CB41 (5'-

TTTAAACTGGATCCAGCCACCATGACCAACAAG-3') (SEQ ID NO 30) /CB55 (5'-

15 CGGCCATAGT

AGCGCTTCAGGTGCAGGGAGCTCATCAGCTTGCCGGTGGTGTTCCTCCTTC-3') (SEQ ID NO 31) and CB42 (SEQ ID 26) / CB86 (5'-

GAAGGAGGACAACACCACCGCAAGCTGATGAGCTCCCTGCACCTGAAGCGCTACT

20 ATGGCC G-3') (SEQ ID NO 32) resulting in two fragments of 446 and 184 base pairs, respectively. These two fragments were assembled in a third PCR with the flanking primers CB41 and CB42. The resulting gene was inserted into the mammalian expression vector pcDNA3.1(-)Hygro/Intron and confirmed by DNA sequencing to have the correct base changes leading to the substitutions F111N and R113T in hINF- $\beta$  (plasmid designated PF085).

To test the activity of the [F111N+ R113T]hINF- $\beta$  variant, PF085 was

25 transfected into the CHO K1 cell line (ATCC #CCL-61) by use of Lipofectamine 2000 (Life Technologies, USA) as transfection agent. 24 hours later the culture medium was harvested and assayed for INF- $\beta$  activity/concentration:

Activity: 56046 IU/ml [primary assay]

ELISA: 80 ng/ml

Specific activity:  $7 \times 10^8$  IU/mg

As seen, the [F111N+R113T]hINF- $\beta$  variant has a very high specific activity,

about twice the specific activity of wt hINF- $\beta$ .

5 EXAMPLE 6. CONSTRUCTION AND EXPRESSION OF INTERFERON  $\beta$  WITH  
ANOTHER INTRODUCED GLYCOSYLATION SITE [Q49N+Q51T]

Analogously to what is described in Example 5 an extra N-linked glycosylation site was introduced in position 49 by means of the substitutions Q49N and Q51T. Using PF043 (*hinf*- $\beta$ /pcDNA3.1 (Invitrogen, USA)) as template, two PCR reactions were performed with two overlapping primer-sets [PBR7 (SEQ ID NO 28) /PBR78 (5'-GGCGTCCTCCTTGGTGAAGTTCTGCAGCTG-3') (SEQ ID NO 33) and PBR8 (5'-ATATATCCCAAGCTTTTATCAGTTGCGCAGGTAGCCGGT-3') (SEQ ID NO 34)/PBR77 (5'-CAGCTGCAGAACTTCACCAAGGAGGACGCC-3') (SEQ ID NO 35) resulting in two fragments of 228 and 369 base pairs, respectively. These two fragments were assembled in a third PCR with the flanking primers PBR7 and PBR8. The resulting gene was inserted into the mammalian expression vector pcDNA3.1(-)Hygro/Intron and confirmed by DNA sequencing to have the correct base changes leading to [Q49N,Q51T]hINF- $\beta$  (plasmid designated PF104).

To test the activity of the [Q49N+Q51T]hINF- $\beta$  variant, PF104 was transfected into the CHO K1 cell line by use of Lipofectamine 2000 (Life Technologies, USA) as transfection agent. 24 hours later the culture medium was harvested and assayed for INF- $\beta$  activity/concentration:

Activity:	17639 IU/ml [primary assay]
ELISA:	10 ng/ml
Specific activity:	$1.7 \times 10^9$ IU/mg

As observed here the [Q49N+Q51T]hINF- $\beta$  variant has a high specific activity. This may be due to poor recognition by one of the monoclonal antibodies used in the ELISA.

EXAMPLE 7. CONSTRUCTION AND EXPRESSION OF INTERFERON  $\beta$  WITH TWO INTRODUCED GLYCOSYLATION SITES

The additional glycosylation sites described in Examples 5 and 6 were introduced into human interferon  $\beta$  by means of the substitutions Q49N, Q51T, F111N, and R113T.

Using PF085 (described in example 5) as template, two PCR reactions were performed with two overlapping primer-sets [PBR89 (5'-CGCGGATCCAGCCACCATGACCAACAAGTGCCTG) (SEQ ID NO 36)/PBR78 (SEQ ID NO 33) and PBR8 (SEQ ID NO 34) /PBR77 (SEQ ID NO 35)] resulting in two fragments of 228 and 369 base pairs, respectively.

5 These two fragments were assembled in a third PCR with the flanking primers PBR89 and PBR8. The resulting gene was inserted into the mammalian expression vector pcDNA3.1(-)Hygro/Intron and confirmed by sequencing to have the correct base changes leading to [Q49N, Q51T, F111N, R113T] hINF- $\beta$  (plasmid designated PF123).

PF123 was transfected into CHO K1 cells by use of Fugene 6 (Roche) as  
10 transfection agent. 24 hours later the culture medium was harvested and assayed for INF- $\beta$  activity/concentration:

Activity: 29401 IU/ml [primary assay]  
ELISA: 14 ng/ml  
Specific activity:  $2.1 \times 10^9$  IU/ml

15 As observed here the [Q49N+Q51T+ F111N+ R113T]hINF- $\beta$  variant also has a high specific activity.

The variant was found to have receptor binding activity in the receptor binding assay described in the Materials and Methods section, which is based on the use of the crosslinking agent DSS.

20 EXAMPLE 8. PRODUCTION OF [Q49N+ Q51T+ F111N+ R113T]INTERFERON- $\beta$  GLYCOSYLATION VARIANT IN ROLLER BOTTLES

A CHOK1 sub-clone (5/G-10) producing the [Q49N+Q51T+F111N+R113T] glycosylation variant was seeded into 2 roller bottles, each with an expanded surface of 1700 cm<sup>2</sup> (Corning, USA), in 200 ml DMEM/F-12 medium (LifeTechnologies; Cat. # 31330) supplemented with 10% FBS and penicillin/streptomycin (P/S). After 2 days the medium was  
25 exchanged. After another 2 days the two roller bottles were nearly 100% confluent and the medium was shifted to 300 ml serum-free UltraCHO medium (BioWhittaker; Cat. # 12-724) supplemented with 1/500 EX-CYTE (Serologicals Proteins; Cat. # 81129N) and P/S. Growing the cells in this medium promotes a higher cell mass, higher than can be achieved in the serum  
30 containing medium. After 2 days the medium was renewed. After another 2 days the medium was shifted to the production medium: DMEM/F-12 medium (Life Technologies; Cat. # 21041) supplemented with 1/100 ITSA (Life Technologies; Cat. # 51300-044) [ITSA stands for Insulin (1.0 g/L) – Transferrin (0.55 g/L) – Selenium (0.67 mg/L) supplement for Adherent cultures], 1/500 EC-CYTE and P/S. In the figure below is shown the production run where 300 ml medium

5 was harvested from each roller bottle every day. The harvested media from the two roller bottles were pooled before a medium sample was taken out for interferon  $\beta$  activity determination. As seen in fig. 2 the production run was terminated after 26 days. After a lag-period of 5 days the activity mediated by the [49N+ Q51T+F111N+R113T]Interferon- $\beta$  variant increased dramatically and for the rest of the production run the harvested interferon- $\beta$  activity per day, in  
10 average, was 2.4 million IU/ml x 600 ml = 1.440 billion IU. In total  $3.2 \times 10^{10}$  IU was produced corresponding to 160 mg protein (with a hypothetical specific activity of  $2 \times 10^8$  IU/mg).

EXAMPLE 9. PRODUCTION, PURIFICATION, AND PEGYLATION OF THE INTERFERON- $\beta$  VARIANT K19R+ K45R+ K123R

To end up with 100 ml serum-free medium containing the Interferon- $\beta$  variant  
15 K19R+K45R+K123R, 3 T-175 flasks were seeded with COS-7 cells in DMEM medium (Life technologies; Cat. # 21969-035) supplemented with 10% FBS plus Glutamine and penicillin/streptomycin. On the day of transfection (at nearly 100% confluency) the medium was renewed with 30 ml fresh medium 4 – 5 hours before the transfection. To prepare the  
20 transfection, 1890  $\mu$ l DMEM medium without supplements was aliquoted into a 14 ml polypropylene tube (Corning). 210  $\mu$ l Fugene 6 (Roche) was added directly into the medium and incubated for 5 min at RT. In the meantime 168  $\mu$ g plasmid DNA ([K19R, K45R, K123R]INF- $\beta$ /pcDNA3.1(-)Hygro; PF #161) was aliquoted into another 14 ml polypropylene tube. After 5 min incubation the Fugene 6 mix was added directly to the DNA solution and incubated for 15 min at RT. After incubation about 700  $\mu$ l was added drop wise to each of the three cell media.

25 Next day the transfection medium was substituted with 35 ml serum-free production medium. The serum-free medium is based on DMEM medium (Life Technologies; Cat. # 31053-028) supplemented with Glutamine, Sodium Pyruvate, penicillin/streptomycin, 1% ITSA (Life Technologies; Cat. # 51300-044), and 0.2% Ex-Cyte (Serologicals Proteins; Cat. # 81-129). Before the production medium was added the cell layers were washed two times in the  
30 DMEM medium without additives.

Three days post-transfection the 100 ml serum-free medium was harvested for purification and PEGylation of the Interferon- $\beta$  variant. pH was adjusted to 6.8 and conductivity adjusted to  $< 10$  mS/cm with Milli Q water. Then the broth was batch adsorbed to 1 ml SP 550 cation exchange resin (TosoHaas) preequilibrated with buffer A (20 mM phosphate, 100 mM

NaCl, pH 7). After 2 h rotation end over end, the resin was allowed to sediment and transferred to a column. The resin was washed with 5 column volumes buffer A and eluted with 2 ml buffer B (20 mM phosphate, 800 mM NaCl, pH 7). The eluate was concentrated to 500 ul on VivaSpin (cutoff 10 kDa) after addition of 5 % ethyleneglycol. The concentrate was adjusted to 50 mM phosphate, 0.3 M NaCl, 20 % ethyleneglycol, pH 8 in a final volume of 2 ml and further concentrated to 0.5 ml.

The final concentrate was PEGylated as follows: to 100 ul of the final concentrate, 25 ul of activated mPEG-SPA (5000 kDa, Shearwater, Alabama) freshly prepared in phosphate buffer, pH 8 were added to make final concentrations of activated PEG of 0, 5, 10, 25 or 50 mg/ml. The reaction was allowed to proceed for 30 min at room temperature and then quenched by addition of 50 mM glycine buffer. Samples were frozen immediately at -80 C and bioactivity was measured as described (Primary Assay). Western blots of each sample were performed in order to evaluate the amount of unreacted Interferon- $\beta$  variant present in the PEGylated sample.

Results demonstrate that at 25 mg activated PEG/ml, nonPEGylated Interferon- $\beta$  variant was absent as judged by western blot and the variant retained 50 % of its bioactivity compared to the control sample (treated identically, but with 0 mg/ml activated PEG).

#### EXAMPLE 10. EXPRESSION AND PURIFICATION OF SOLUBLE IFNAR2

The cDNA's encoding the extracellular domain of IFNAR-1 and IFNAR-2 (termed IFNAR1ec and IFNAR2ec, respectively) were amplified from HeLa cell cDNA using PCR with primers corresponding to the first 10 amino acid residues and the final 10 amino acid residues of the extracellular domain of IFNAR-2 (the nucleotide sequence of which is apparent from Novick et al., Cell, Vol. 77, pp 391-400, 1994) and the first 10 amino acid residues and the final 10 amino acid residues of the extracellular domain of IFNAR-1 (the nucleotide sequence of which is apparent from Uze et al., Cell Vol. 60, 225-234, 1990). The cDNA's were subcloned into the pBlueBac 4.5/V5-His-TOPO vector (Invitrogen) and a recombinant Baculovirus obtained by homologous recombination, plaque purification, and propagation in Sf9 cells. Sf9 cells were infected with the recombinant Baculovirus and expression from the resulting cells was obtained essentially as described in Example 2.

IFNAR1ec and IFNAR2ec protein was observed in culture supernatants two to three days after infection of Sf9 cells with recombinant baculovirus. The activity of soluble receptors was observed in an Interferon antagonist assay. Briefly, Hela cells containing the ISRE element (as described in the Primary Assay above) are stimulated with a sub-maximal dose of human wild-type Interferon  $\beta$  in the presence of varying concentrations of IFNARec supernatant. The antagonist effect of the supernatant is directly proportional to the amount of soluble receptor present.

IFNAR2ec was purified from filtered culture supernatants using ion exchange, and affinity chromatography. Culture supernatants positive for IFNAR2ec were pH adjusted to 7.5 and loaded onto an anion exchange column, and the bound recombinant protein was eluted using 500mM NaCl. The partially pure IFNAR2ec was then diluted and pH adjusted to 8.0, before further purification using binding to a TALON™ Metal Affinity Resin and elution with imidazol. The final preparation was frozen in aliquots. IFNAR1ec can be purified as described for IFNAR2ec with the exception that cation exchange chromatography at pH6.0 will be used as the ion exchange step.

#### EXAMPLE 11. USE OF SOLUBLE IFNAR2 FOR PURIFICATION AND PEGYLATION OF INTERFERON- $\beta$ AND VARIANTS THEREOF

Purified IFNAR2 obtained as described in Example 9 is immobilized either through amino or carboxyl groups using e.g. CNBr-activated Sepharose 4B or EAH Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech, Affinity Chromatography, Principles and Methods, 18-1022-29, edition AB). It is critically important that the coupling method allows functional IFNAR2 to be immobilized and this is tested through optimization of the coupling conditions (pH, coupling buffer, ratio of IFNAR 2 to activate matrix etc). Another critical parameter is the blocking of excess active groups. Subsequently, testing of binding capacity by addition of interferon- $\beta$  and measurement of breakthrough is carried out.

Optimally immobilized IFNAR2 is used for purification of Interferon- $\beta$  as follows. A 5 ml column with 1 mg IFNAR 2 immobilized per ml gel is equilibrated with buffer A (20 mM phosphate, 300 mM NaCl, pH 7). Then the column is loaded with a 2 mg Interferon- $\beta$  sample in buffer A and subsequently washed with 5 column volumes buffer A. Elution is obtained by pumping 2 column volumes of buffer B onto the column. Fractions of 1 ml are collected and assayed for bioactivity. Optimal elution conditions are dependent on the

5 immobilization method, but examples of elution conditions include pH 1.5 – 3 (e.g. 0.1 M glycine pH 2.3 in 0.5 M NaCl), pH 11.5 – 12, 3.5 M MgCl<sub>2</sub>, 6M urea or the like.

EXAMPLE 12. USE OF IMMOBILIZED IFNAR2 FOR PEGYLATION OF INTERFERON  $\beta$  (VARIANTS)

10 In addition to the use described in Example 10, immobilized IFNAR 2 may be used for optimal PEGylation, wherein PEGylation of the part of Interferon- $\beta$  or variants thereof interacting with the receptor is avoided.

15 A 5 ml column with 1 mg IFNAR 2 immobilized per ml gel is equilibrated with buffer A (20 mM phosphate, 300 mM NaCl, pH 7). Then the column is loaded with a 2 mg Interferon- $\beta$  sample in buffer A and subsequently washed with 5 column volumes buffer A. A solution of activated mPEG-SPA (1–50 mg/ml in buffer A) is pumped on the column and allowed to react for 15 min–12 h depending on temperature. One preferred range of combination of residence time and temperature is 15–60 min, 10–20 °C, another is 30 min to 5 h, 2–8°C . After the indicated time period, elution is obtained by pumping 2 column volumes of buffer B onto the column. Fractions of 1 ml are collected and assayed for bioactivity using the primary screening assay. Optimal elution conditions are dependent on the immobilization method, but  
20 examples of elution conditions include pH 1.5–3 (e.g. 0.1 M glycine pH 2.3 in 0.5 M NaCl), pH 11.5–12, 3.5 M MgCl<sub>2</sub>, 6M Urea or the like.

EXAMPLE 13. ANTIVIRAL ACTIVITY OF PEGYLATED VARIANT

25 The pegylated IFN- $\beta$  variant protein, K19R+K45R+K123R, was assayed using the antiviral bioassay. Wild-type and variant proteins were added to A549 cells in concentrations from 10<sup>-10</sup>–0.0001 IU/mL in triplicate cultures.

The pegylated IFN- $\beta$  variant showed total inhibition of EMC virus induced cell death at a concentration of 3 IU/mL, with an EC<sub>50</sub> of 0.13 IU/mL (Figure 1). The wild-type standard shows virus inhibition with an EC<sub>50</sub> of 1.4 IU/mL.

30 These results demonstrate that the pegylation of the modified interferon  $\beta$  polypeptide resulted in a conjugate with full anti-viral activity.

#### EXAMPLE 14. ANTIBODY NEUTRALISATION OF GLYCOSYLATED VARIANT

The antibody neutralisation of wild-type and glycosylated IFN- $\beta$  variant protein, Q49N+Q51T+F111N+R113T, was assayed using the ISRE neutralisation assay. Interferon  $\beta$  wild-type and variant proteins (in five fold dilutions starting at 12500 IU/mL) were incubated with polyclonal rabbit anti-interferon  $\beta$  antibody (PBL Biomedical Laboratories) in concentrations 0, 40 and 200 ng/mL.

In the presence of 200 ng/mL polyclonal rabbit anti-serum the activity of the wild type interferon  $\beta$  protein was reduced 11.8 times whereas the activity of the glycosylated interferon  $\beta$  variant only was reduced 3.0 times. Thus the degree of antibody recognition of the interferon  $\beta$  variant was reduced by 75% of the wt level, see Table 1 below. These results demonstrate that the recognition of the glycosylated mutant interferon  $\beta$  by polyclonal antibodies raised in animals immunised with wild-type human interferon  $\beta$  is highly reduced. Thus, a large portion of the immunogenic epitopes in wild-type human interferon  $\beta$  have been removed/shielded by the modifications made in the variant molecule.

Table 1

Antibody conc. (ng/mL)	Protein	EC50	Fold inhibition	Reduction of antibody neutralisation
0	wt	0.00039	-	-
	variant	0.00020	-	-
40	wt	0.00190	4.8	-
	variant	0.00020	1.0	79%
200	wt	0.00461	11.8	-
	variant	0.00059	3.0	75%

#### EXAMPLE 15. CONSTRUCTION AND EXPRESSION OF INTERFERON $\beta$ MOLECULES WITH MODIFIED N-TERMINAL

N-terminally modified variants of interferon  $\beta$  were constructed as described in the preceding examples.

For the construction of an expression plasmid for the interferon  $\beta$  variant, INFB S(-1)A + M1Q the following primers were used:

CBProFpr110:

AAC TGG ATC CAG CCA CCA TGA CCA ACA AGT GCC TGC TCC AGA TCG CCC TGC TCC TGT GCT TCA GCA CCA CGG CCC TAG CCC AGA GCT AC (SEQ ID NO 37) and

CBProFpr42 (SEQ ID NO 26),



5 For the construction of an expression plasmid for the interferon variant, INF $\beta$  S(-1)AQ (indicating substitution of the S residue located in position (-1) with an A and a Q residue) the following primers were used:

CBProFpr109:

AAC TGG ATC CAG CCA CCA TGA CCA ACA AGT GCC TGC TCC AGA TCG CCC TGC

10 TCC TGT GCT TCA GCA CCA CGG CCC TAG CCC AGA TGA GCT AC (SEQ ID NO 38)

and

CBProFpr42 (SEQ ID NO 26).

To test the activity of these variants the respective plasmids; pF154 and pF163 were transfected into CHO K1 cells using Lipofectamine 2000 (Life Technologies, USA) as  
15 transfection reagent. The supernatants were harvested 24 hours post transfection and assayed in the primary activity assay and in the ELISA as described in the Materials and Methods section.

The following results were obtained:

INFB S-1A + M1Q (pF154):

Activity: 106410 IU/ml  
20 ELISA: 333 ng/ml  
Specific activity:  $3.2 \times 10^8$  IU/mg

INFB S-1AQ (pF163):

Activity: 90634 IU/ml  
25 ELISA: 193 ng/ml  
Specific activity:  $4.7 \times 10^8$  IU/mg

These molecules are as active as wild type human interferon  $\beta$ .

#### EXAMPLE 16. PREPARATION OF PEGYLATED IFN- $\beta$ VARIANTS

50 microliters of a 0.3 mg/ml solution of recombinant human IFN- $\beta$  polypeptide comprising the mutations Q49N+Q51T+ K19R+ K45R+ K123R in 50 mM Na-acetate, 35%  
30 ethylene glycol, pH 5.5 were mixed with 10  $\mu$ l 0.5 M Na-phosphate, pH 8.0 and 20  $\mu$ l 50 mM Na-phosphate, 0.1 M NaCl, 30% ethylene glycol, pH 8.0 containing 0.02 mg/ml SPA-mPEG (N-succinimidyl Propionate methoxy polyethylene glycol). This corresponds to a 10 molar excess of SPA-mPEG to IFN- $\beta$ .

After  $\frac{1}{2}$  hour with gentle rotation at room temperature, the reaction was quenched  
35 by addition of 5  $\mu$ l 20 mM Glycine, pH 8.0. At this stage, the reaction mixture contained a mixture of unmodified as well as pegylated forms of recombinant human IFN- $\beta$ .

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Activity:

*In vitro* testing using the primary screening assay demonstrated that the pegylated material retained 40 % activity, as compared to the unmodified recombinant human IFN- $\beta$ .

10 In another experiment, 50  $\mu$ l of a 0.14 mg/ml solution of recombinant human IFN  $\beta$  polypeptide comprising the mutations Q49N+Q51T in 50 mM Na-acetate, 35% ethylene glycol, pH 5.5 was mixed with 10  $\mu$ l 0.5 M Na-phosphate, pH 8.0 and 20  $\mu$ l 50 mM Na-phosphate, 0.1 M NaCl, 30% ethylene glycol, pH 8.0 containing 0.03 mg/ml SPA-mPEG. This gave a 10 molar excess of SPA-mPEG to IFN- $\beta$ .

15 After  $\frac{1}{2}$  hour with gentle rotation at room temperature, the reaction was quenched by addition of 5  $\mu$ l 20 mM Glycine, pH 8.0. At this stage, the reaction mixture contained a mixture of unmodified as well as pegylated forms of recombinant human IFN- $\beta$ .

Activity:

*In vitro* testing using the primary screening assay demonstrated that the pegylated material retained 20 % activity, as compared to the unmodified recombinant human IFN- $\beta$ .

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While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques, methods, compositions, apparatus and systems described above may be used in various combinations. *All publications, patents, patent applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.*